Evidence that genome editing is superior to transgenesis for enhancing animal traits

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Supplementary Figures

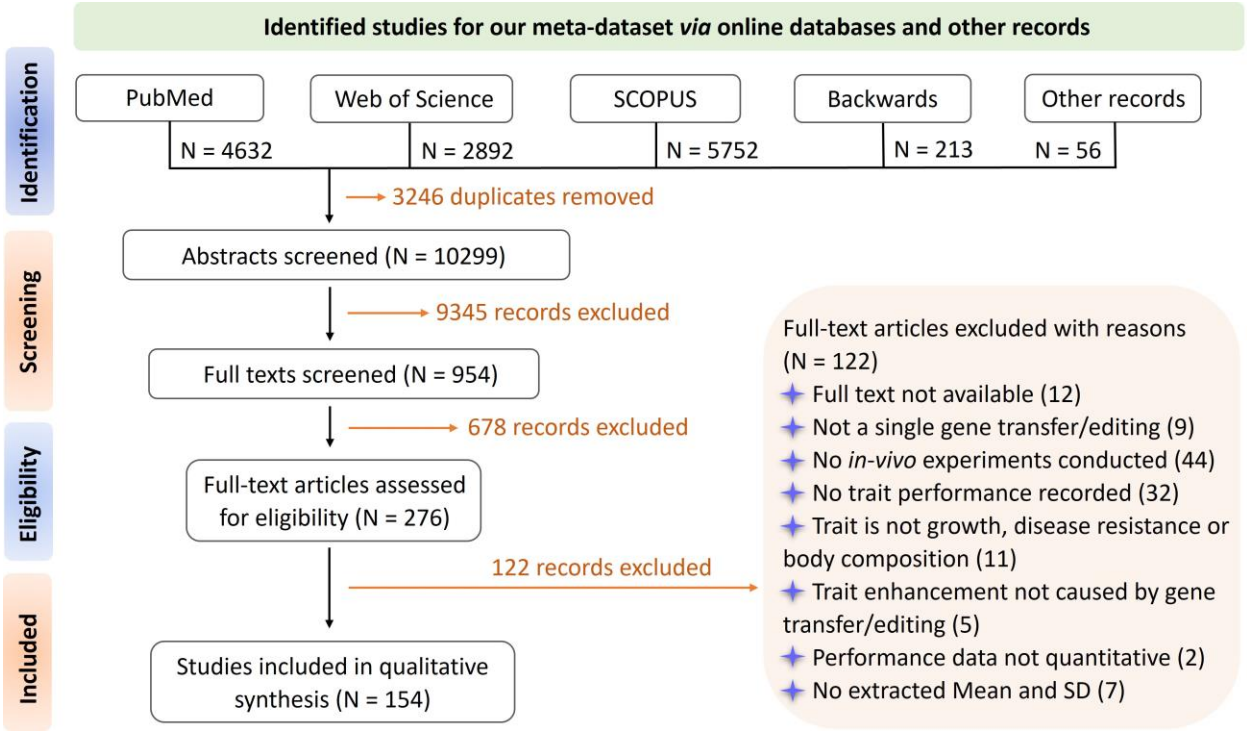


Fig. 1 PRISMA diagram describing the search results in different search engines and the different steps of selecting articles for inclusion in the meta-analysis. Depicted are the number of studies excluded at each stage and then those extracted, screened, and included in the meta-analysis.

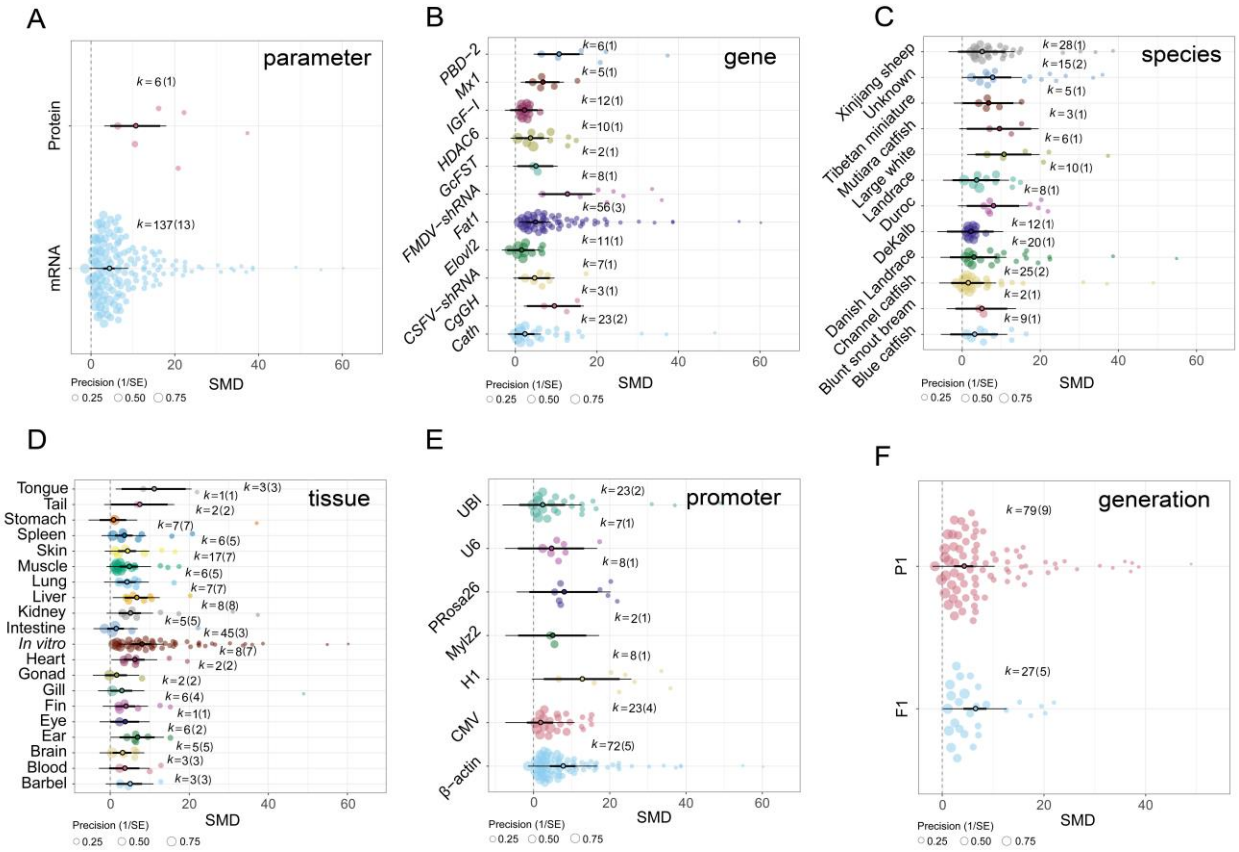


Fig. 2 Significant induction of transgenes was observed across different moderators. A The orchard plot showed significant mRNA and protein expression of transgenes. **B** Different mRNA/protein expression levels were found across transgenes. **C** The effect of transgenesis on gene/protein expression is species-specific based on a species-moderator analysis. **D** The mRNA/protein expression was tissue-specific according to a tissue-moderator analysis. **E** The mRNA levels and protein expression of transgenes were significantly different across promoters. **F** Significant mRNA and protein expression was detected in both P1 and F1 generations. The *x*-axis shows the values of effect sizes as standardized mean difference (SMD), while the *y*-axis represents the density distribution of effect sizes. The size of the dots represents the precision of each effect size (1/SE). Thick black whisker lines and thin black lines represent 95% confidence intervals (CIs) and 95% prediction intervals, respectively. A bold error bar (95% CI) shows whether the overall effect size is significantly ($P < 0.05$) different from zero (i.e. not overlapping zero). *k* is the number of effect sizes and the number of studies (the number in brackets).

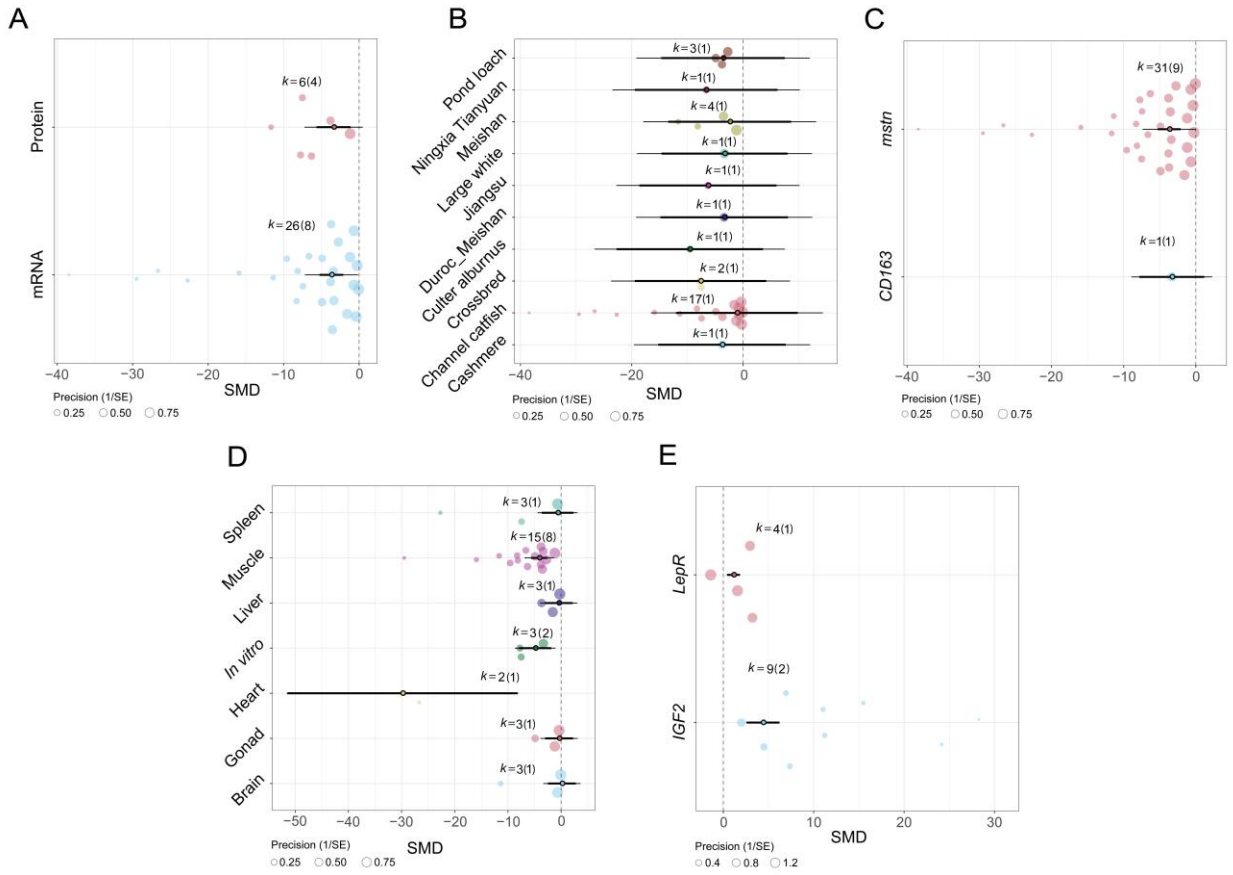


Fig. 3 Significant inhibition of innate genes by gene editing was observed across different moderators. **A** The orchard plot showed significantly reduced mRNA and protein expression of innate genes using gene editing. **B-D** Differential downregulation of mRNA/protein expression levels was found across species, gene and tissue. **C** The mRNA levels and protein expression of innate genes were significantly induced when the *LepR* and *IGF2* were edited. The *x*-axis shows the values of effect sizes as standardized mean difference (SMD), while the *y*-axis represents the density distribution of effect sizes. The size of the dots represents the precision of each effect size (1/SE). Thick black whisker lines and thin black lines represent 95% confidence intervals (CIs) and 95% prediction intervals, respectively. A bold error bar (95% CI) shows whether the overall effect size is significantly ($P < 0.05$) different from zero (i.e. not overlapping zero). *k* is the number of effect sizes and the number of studies (the number in brackets).

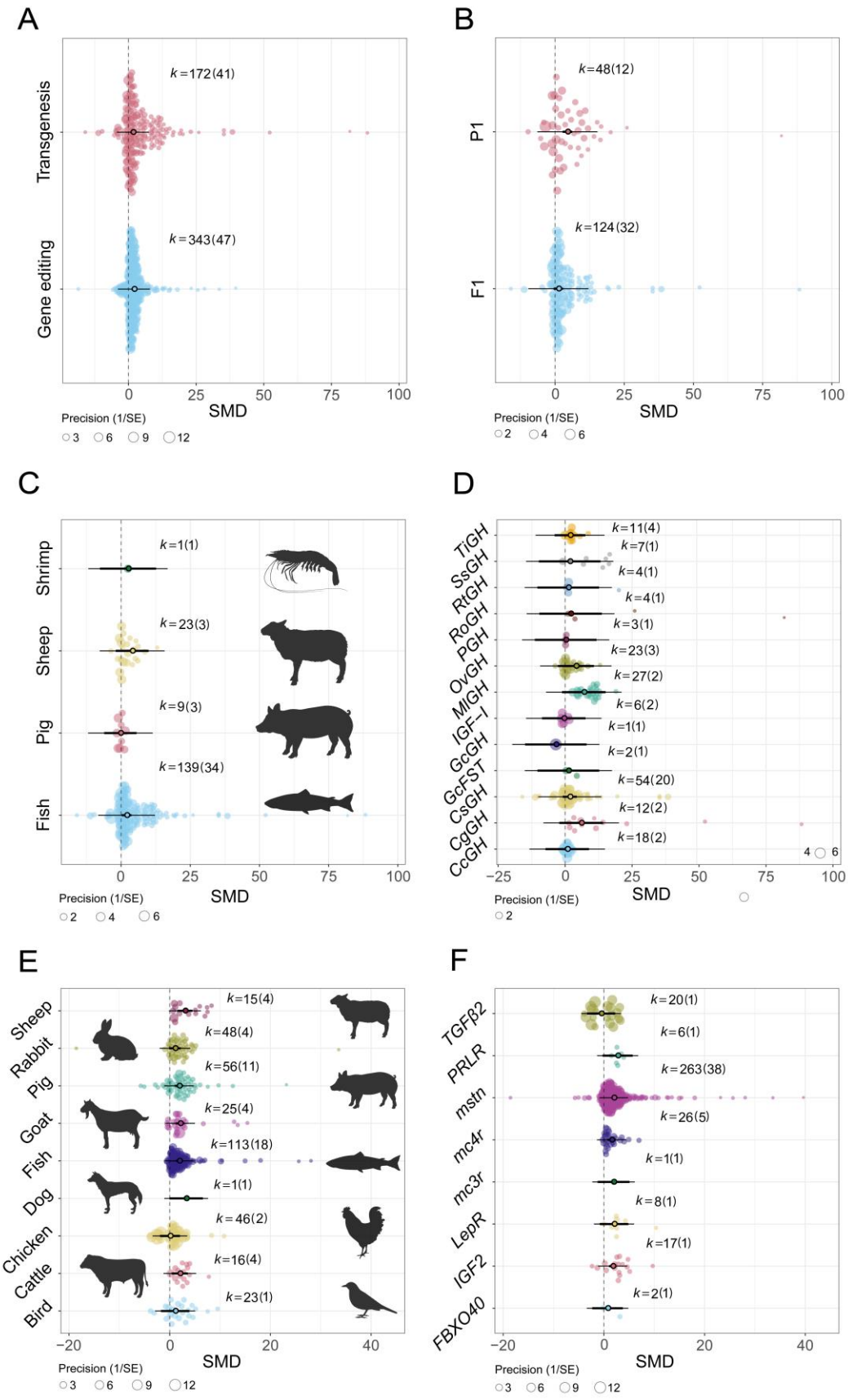

A
Transgenesis
Gene editing
k = 172(41)
k = 343(47)
0
25
50
75
100
SMD
Precision (1/SE)
3
6
9
12
B
P1
F1
k = 48(12)
k = 124(32)
0
25
50
75
100
SMD
Precision (1/SE)
2
4
6
C
Shrimp
Sheep
Pig
Fish
k = 1(1)
k = 23(3)
k = 9(3)
k = 139(34)
0
25
50
75
100
SMD
Precision (1/SE)
2
4
6
D
TiGH
SsGH
RtGH
RoGH
PGH
OvGH
MIGH
IGF-I
GcGH
GcFST
CsGH
CgGH
CcGH
k = 11(4)
k = 7(1)
k = 4(1)
k = 4(1)
k = 3(1)
k = 23(3)
k = 27(2)
k = 6(2)
k = 1(1)
k = 2(1)
k = 54(20)
k = 12(2)
k = 18(2)
4
6
−25
0
25
50
75
100
SMD
Precision (1/SE)
2
E
Sheep
Rabbit
Pig
Goat
Fish
Dog
Chicken
Cattle
Bird
k = 15(4)
k = 48(4)
k = 56(11)
k = 25(4)
k = 113(18)
k = 1(1)
k = 46(2)
k = 16(4)
k = 23(1)
−20
0
20
40
SMD
Precision (1/SE)
3
6
9
12
F
TGFβ2
PRLR
mstn
mc4r
mc3r
LepR
IGF2
FBXO40
k = 20(1)
k = 6(1)
k = 263(38)
k = 26(5)
k = 1(1)
k = 8(1)
k = 17(1)
k = 2(1)
−20
0
20
40
SMD
Precision (1/SE)
3
6
9
12

Fig. 4 Comparison of effects of transgenesis and gene editing on growth enhancement in farmed animals across moderators. A The orchard plot showed both transgenesis and gene editing had positive effects on growth enhancement. **B** The positive effect of transgenesis and gene editing on growth was observed in both P1 and F1 generations. **C** The effect of transgenesis on growth traits was determined across 4 taxonomic classes with different effect sizes. **D** The effect of transgenesis on growth traits was gene-specific based on a gene-moderator analysis. **E** Gene editing showed a larger effect on growth traits as reflected by a border taxon involved combining all growth-related parameters. **F** The effect of gene editing on growth traits was gene-specific based on a gene-moderator analysis. The *x*-axis shows the values of effect sizes as standardized mean difference (SMD), while the *y*-axis represents the density distribution of effect sizes. The size of the dots represents the precision of each effect size (1/SE). Thick black whisker lines and thin black lines represent 95% confidence intervals (CIs) and 95% prediction intervals, respectively. A bold error bar (95% CI) shows whether the overall effect size is significantly ($P < 0.05$) different from zero (i.e. not overlapping zero). *k* is the number of effect sizes and the number of studies (the number in brackets).

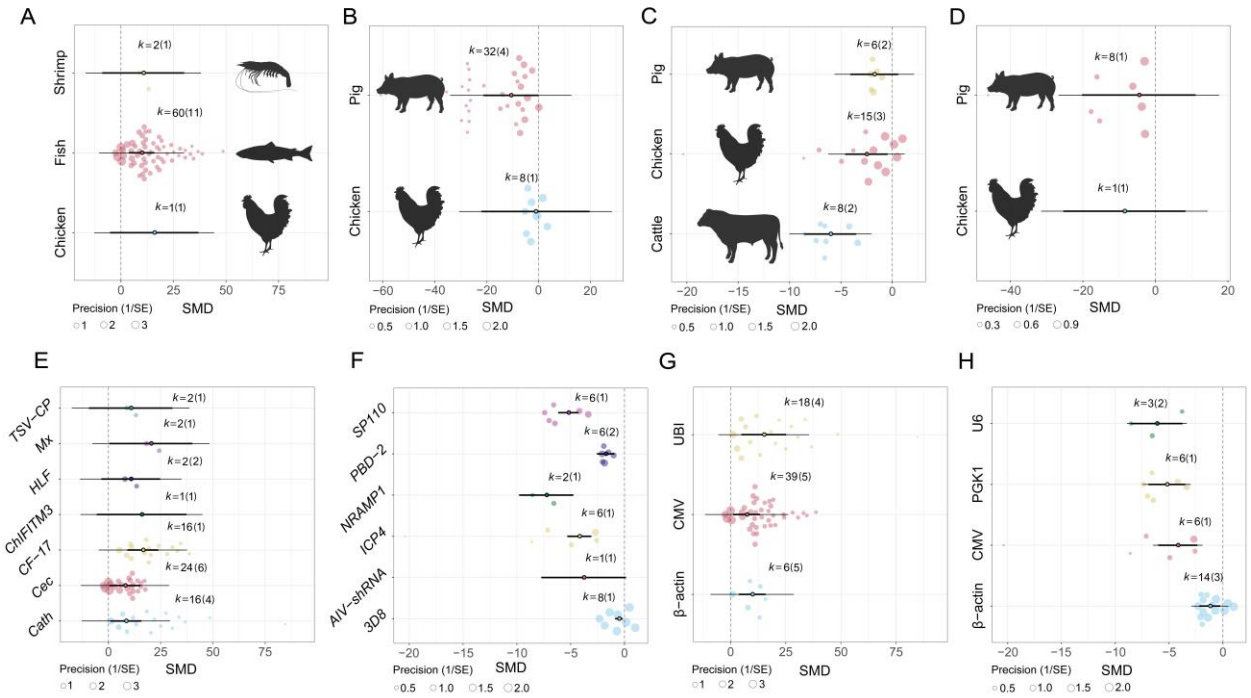


Fig. 5 The effect of transgenesis on disease resistance enhancement in farmed animals across moderators. A-D Transgenesis had different effects on CSR, virus RNA, pathogen load and virus titer, respectively, based on taxon-moderator analysis. **E-F** Different transgenes showed various effects on CSR and pathogen load, respectively, based on the gene-moderator analysis. **G-H** Different promoters showed various effects on CSR and pathogen load, respectively, based on the promoter-moderator analysis. The *x*-axis shows the values of effect sizes as standardized mean difference (SMD), while the *y*-axis represents the density distribution of effect sizes. The size of the dots represents the precision of each effect size (1/SE). Thick black whisker lines and thin black lines represent 95% confidence intervals (CIs) and 95% prediction intervals, respectively. A bold error bar (95% CI) shows whether the overall effect size is significantly ($P < 0.05$) different from zero (i.e. not overlapping zero). *k* is the number of effect sizes and the number of studies (the number in brackets). CSR, cumulative survival rate.

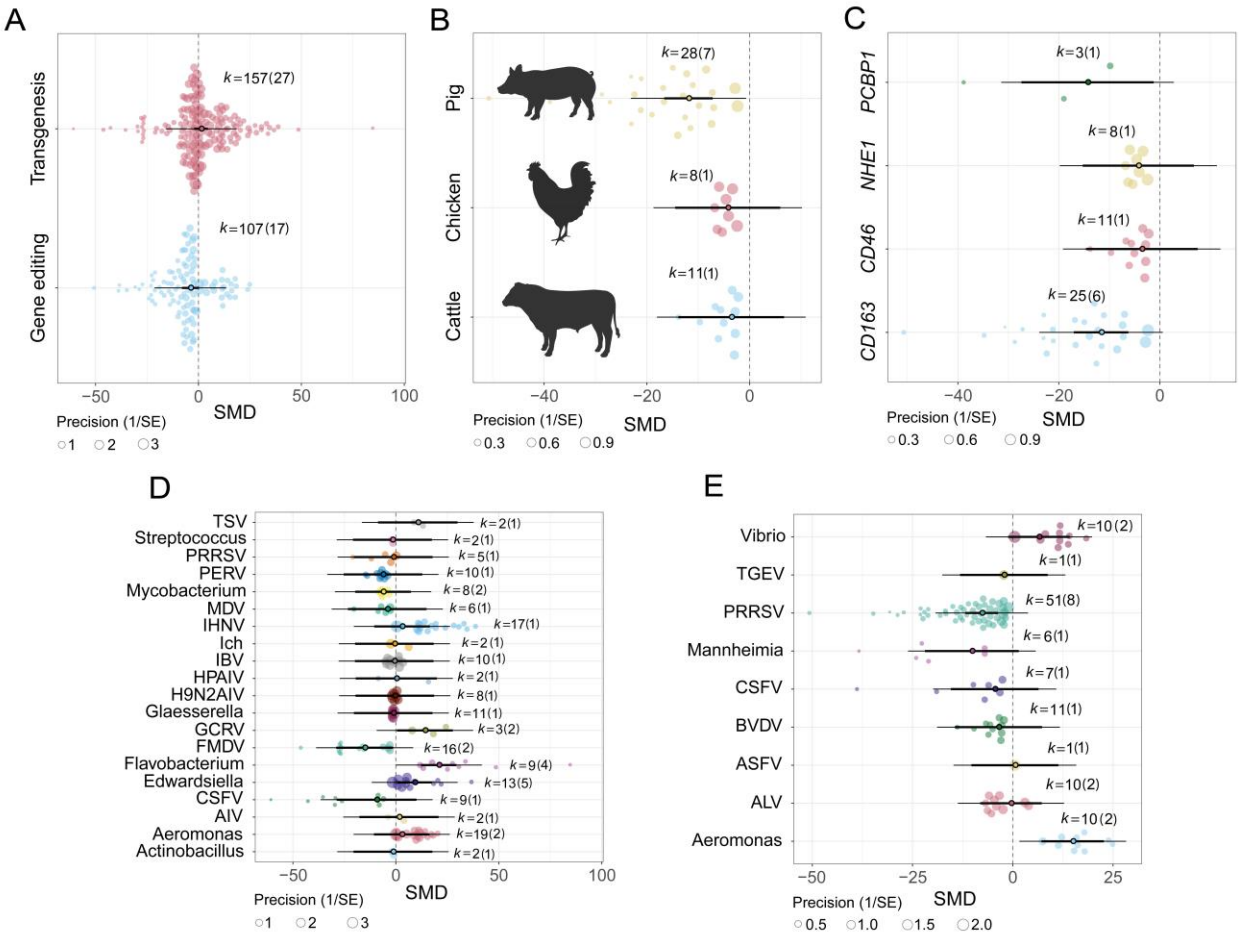


Fig. 6 The effect of gene editing on disease resistance enhancement in farmed animals across moderators. A Comparison of the effect of transgenesis and gene editing on disease resistance using a full model. **B** The effect of gene editing on virus RNA across taxonomic classes. **C** The effect of gene editing on virus RNA across genes. **D-E** The effect of transgenesis and gene editing on the enhancement of disease resistance across pathogens. The *x*-axis shows the values of effect sizes as standardized mean difference (SMD), while the *y*-axis represents the density distribution of effect sizes. The size of the dots represents the precision of each effect size (1/SE). Thick black whisker lines and thin black lines represent 95% confidence intervals (CIs) and 95% prediction intervals, respectively. A bold error bar (95% CI) shows whether the overall effect size is significantly ($P < 0.05$) different from zero (i.e. not overlapping zero). *k* is the number of effect sizes and the number of studies (the number in brackets).

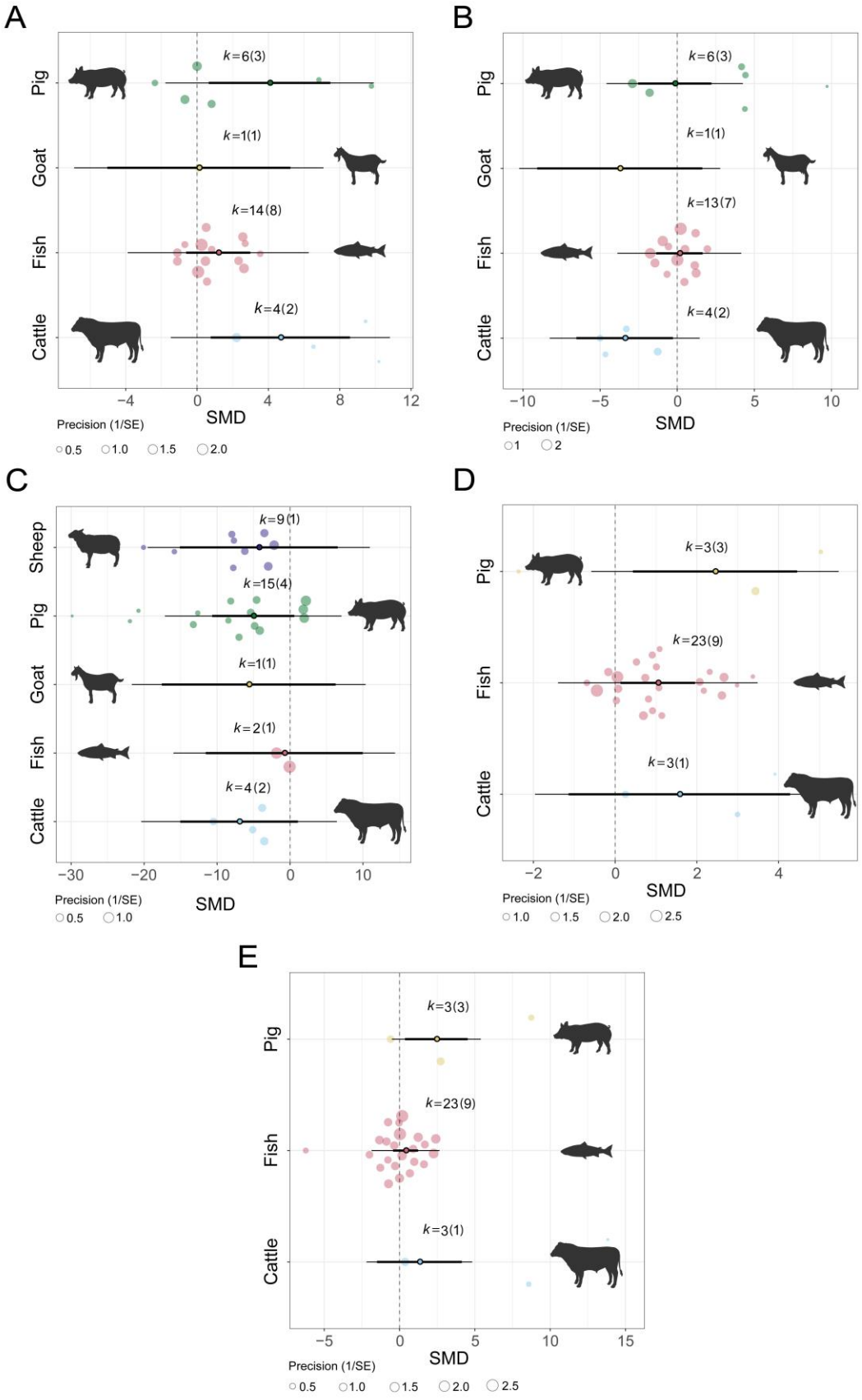

A
Pig
Goat
Fish
Cattle
k=6(3)
k=1(1)
k=14(8)
k=4(2)
−4
0
4
8
12
SMD
Precision (1/SE)
0.5
1.0
1.5
2.0
B
Pig
Goat
Fish
Cattle
k=6(3)
k=1(1)
k=13(7)
k=4(2)
−10
−5
0
5
10
SMD
Precision (1/SE)
1
2
C
Sheep
Pig
Goat
Fish
Cattle
k=9(1)
k=15(4)
k=1(1)
k=2(1)
k=4(2)
−30
−20
−10
0
10
SMD
Precision (1/SE)
0.5
1.0
D
Pig
Fish
Cattle
k=3(3)
k=23(9)
k=3(1)
−2
0
2
4
SMD
Precision (1/SE)
1.0
1.5
2.0
2.5
E
Pig
Fish
Cattle
k=3(3)
k=23(9)
k=3(1)
−5
0
5
10
15
SMD
Precision (1/SE)
0.5
1.0
1.5
2.0
2.5

Fig. 7 The effect of transgenesis on fatty acid across taxa based on different parameters in farmed animals. The effects of transgenesis on ω-3 (**A**), ω-6 (**B**), ω-6/ω-3 (**C**), DHA (**D**), and EPA (**E**) across different taxa. The *x*-axis shows the values of effect sizes as standardized mean difference (SMD), while the *y*-axis represents the density distribution of effect sizes. The size of the dots represents the precision of each effect size (1/SE). Thick black whisker lines and thin black lines represent 95% confidence intervals (CIs) and 95% prediction intervals, respectively. A bold error bar (95% CI) shows whether the overall effect size is significantly ($P < 0.05$) different from zero (i.e. not overlapping zero). *k* is the number of effect sizes and the number of studies (the number in brackets). DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; ω-3, *n*-3 polyunsaturated fatty acid; ω-6, *n*-6 polyunsaturated fatty acid; ω-6/ω-3, the ratio of ω-6/ω-3.

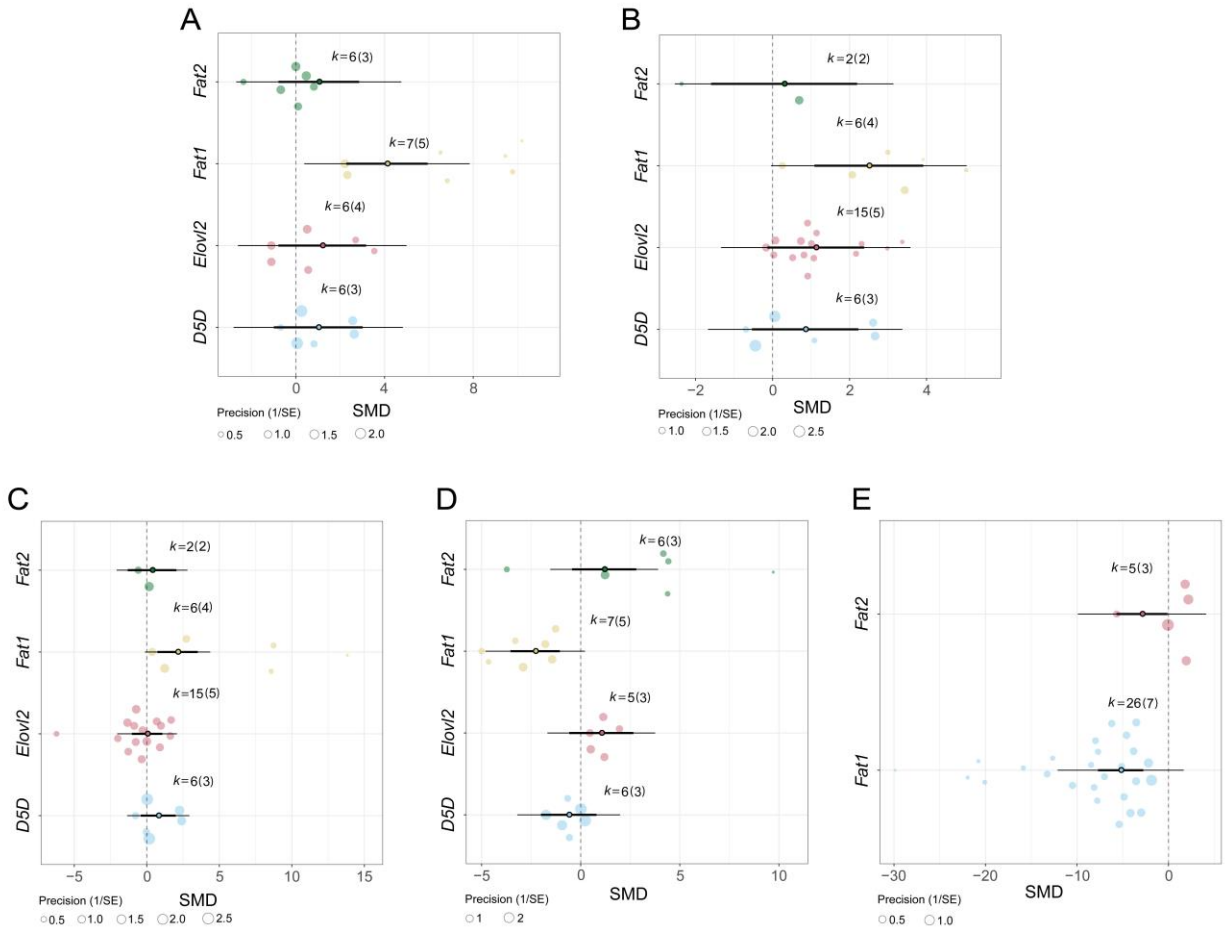


Fig. 8 The effect of transgenesis on fatty acid across genes based on different parameters in farmed animals. The effects of transgenesis on ω-3 (**A**), DHA (**B**), EPA (**C**), ω-6 (**D**), and ω-6/ω-3 (**E**) across different genes. The *x*-axis shows the values of effect sizes as standardized mean difference (SMD), while the *y*-axis represents the density distribution of effect sizes. The size of the dots represents the precision of each effect size (1/SE). Thick black whisker lines and thin black lines represent 95% confidence intervals (CIs) and 95% prediction intervals, respectively. A bold error bar (95% CI) shows whether the overall effect size is significantly ($P < 0.05$) different from zero (i.e. not overlapping zero). *k* is the number of effect sizes and the number of studies (the number in brackets).

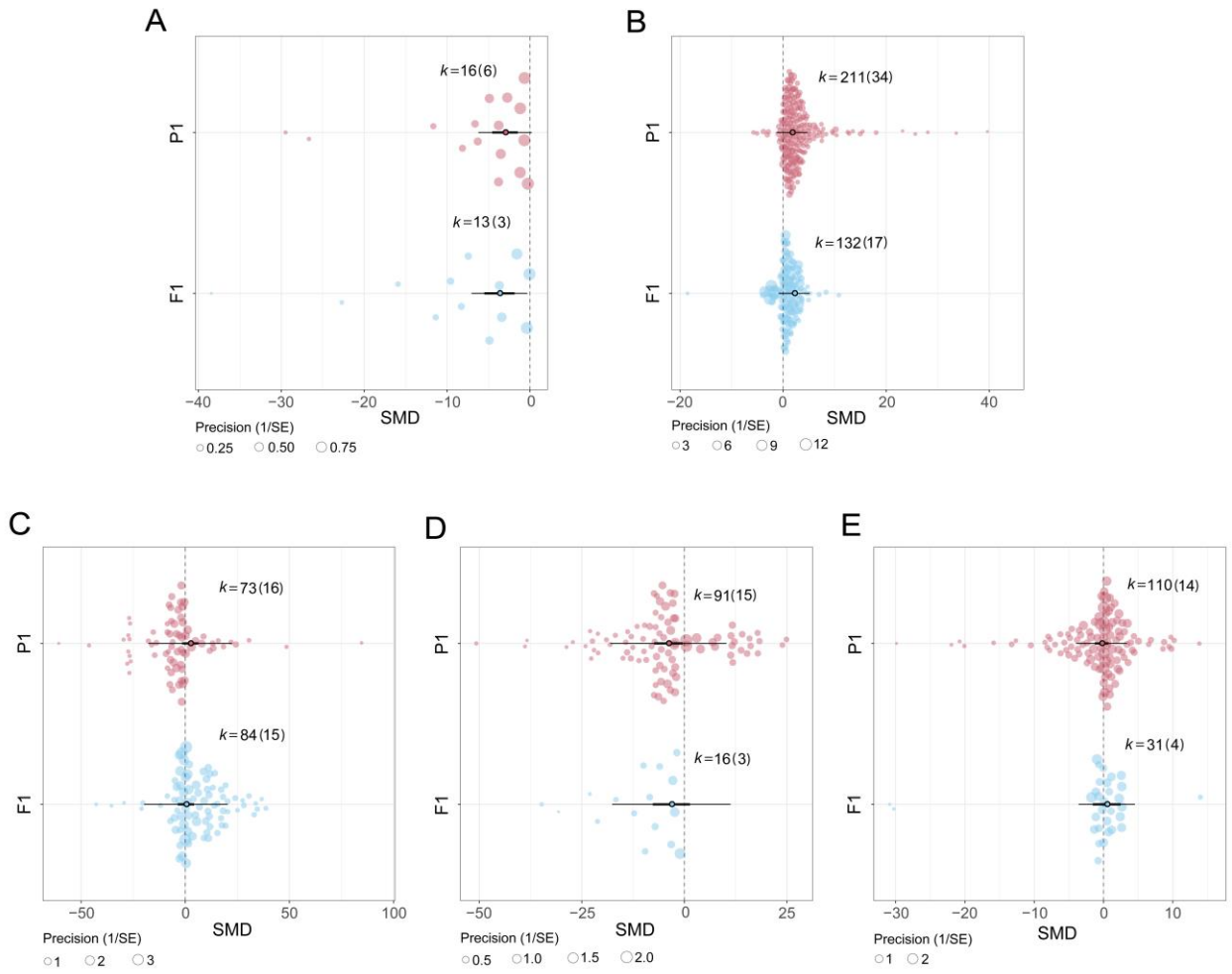
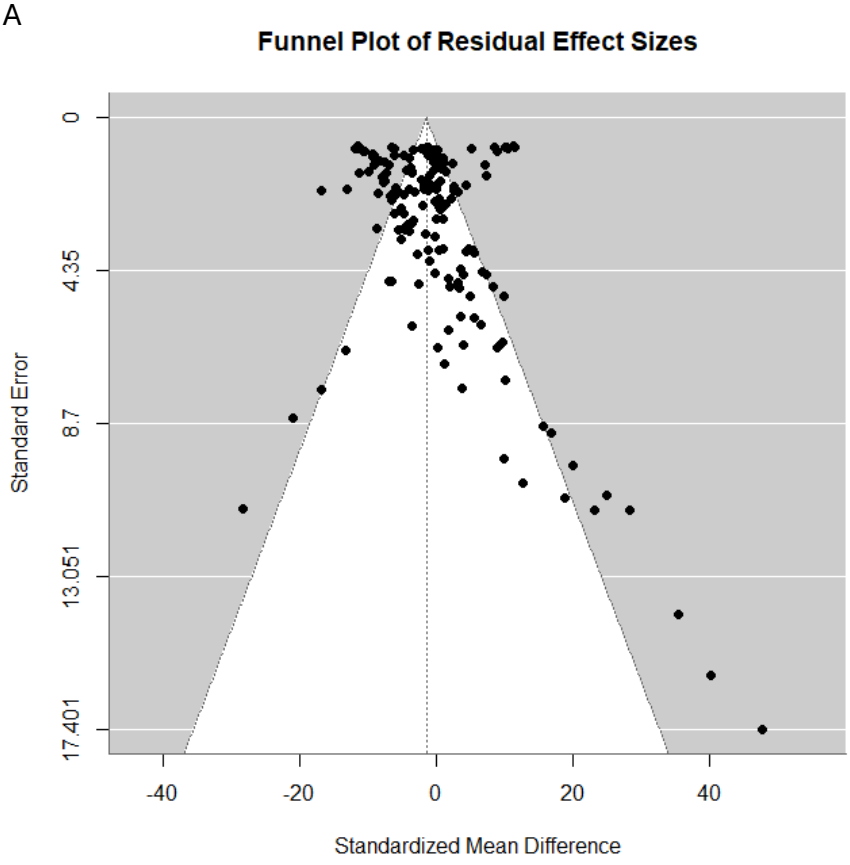
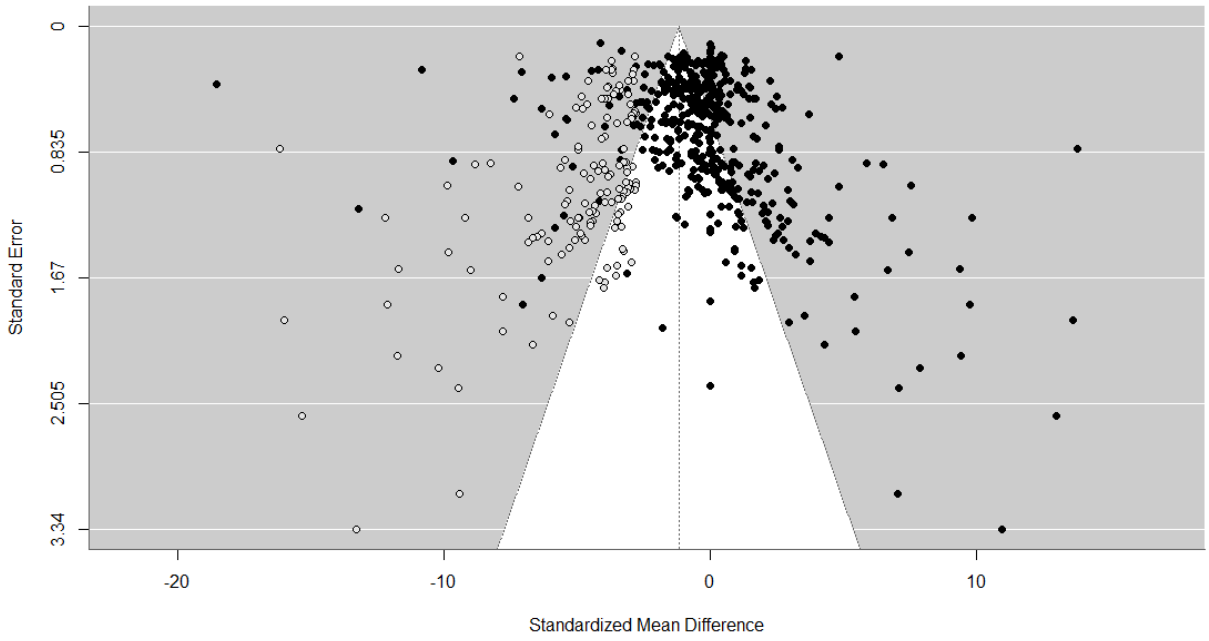


Fig. 9 The effect of transgenesis and gene editing on trait performance across generations (P1 and F1) in farmed animals. A-B The effect of gene editing on gene/protein expression and growth across generations. **C-D** The effect of transgenesis and gene editing on disease resistance across generations. **E** The effect of transgenesis on fatty acid across generations. The *x*-axis shows the values of effect sizes as standardized mean difference (SMD), while the *y*-axis represents the density distribution of effect sizes. The size of the dots represents the precision of each effect size (1/SE). Thick black whisker lines and thin black lines represent 95% confidence intervals (CIs) and 95% prediction intervals, respectively. A bold error bar (95% CI) shows whether the overall effect size is significantly ($P < 0.05$) different from zero (i.e. not overlapping zero). *k* is the number of effect sizes and the number of studies (the number in brackets).

A


Funnel Plot of Residual Effect Sizes
Standard Error
0
4.35
8.7
13.051
17.401
-40
-20
0
20
40
Standardized Mean Difference

B


Standard Error
0
0.835
1.67
2.505
3.34
-20
-10
0
10
Standardized Mean Difference

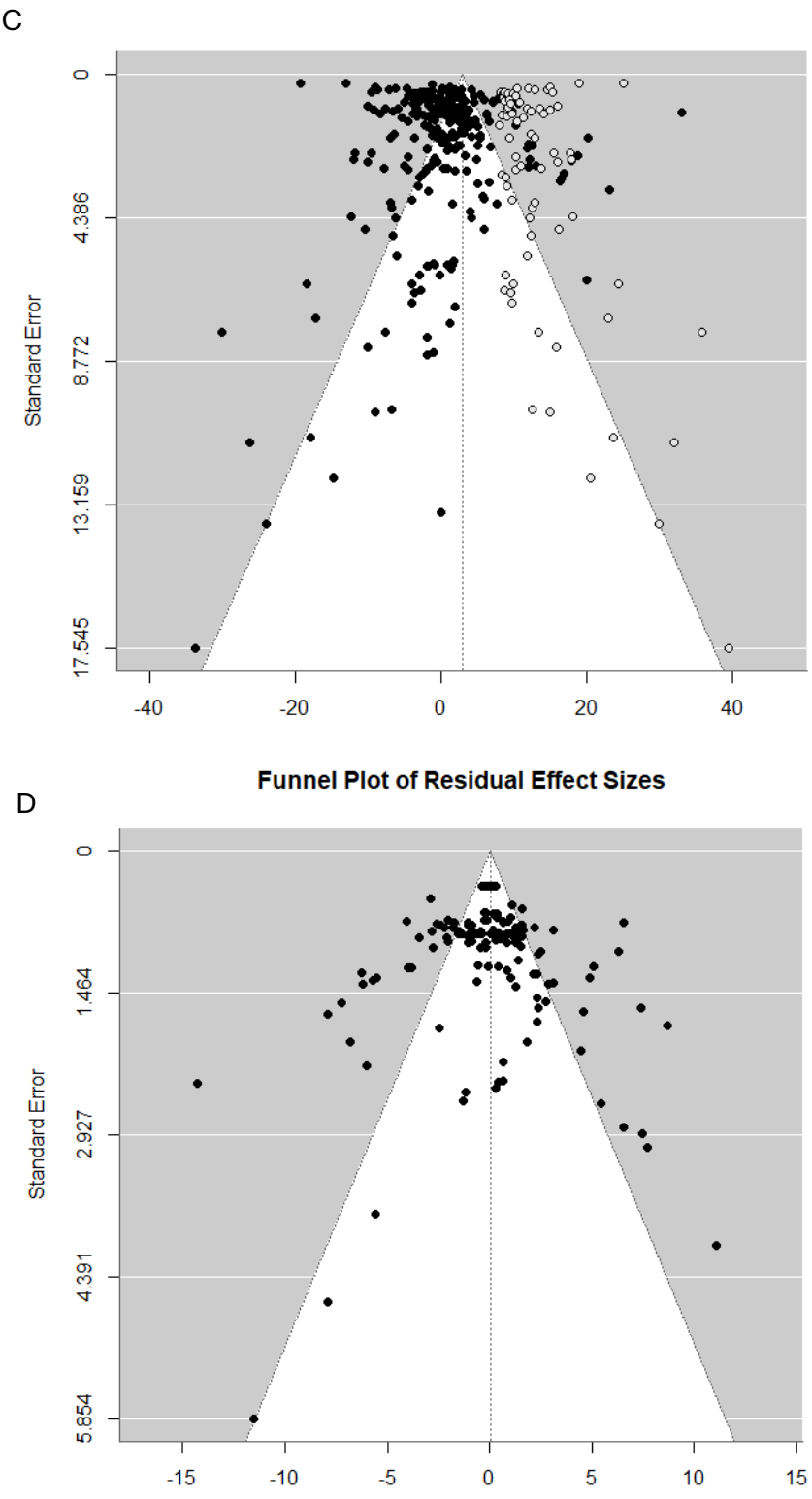
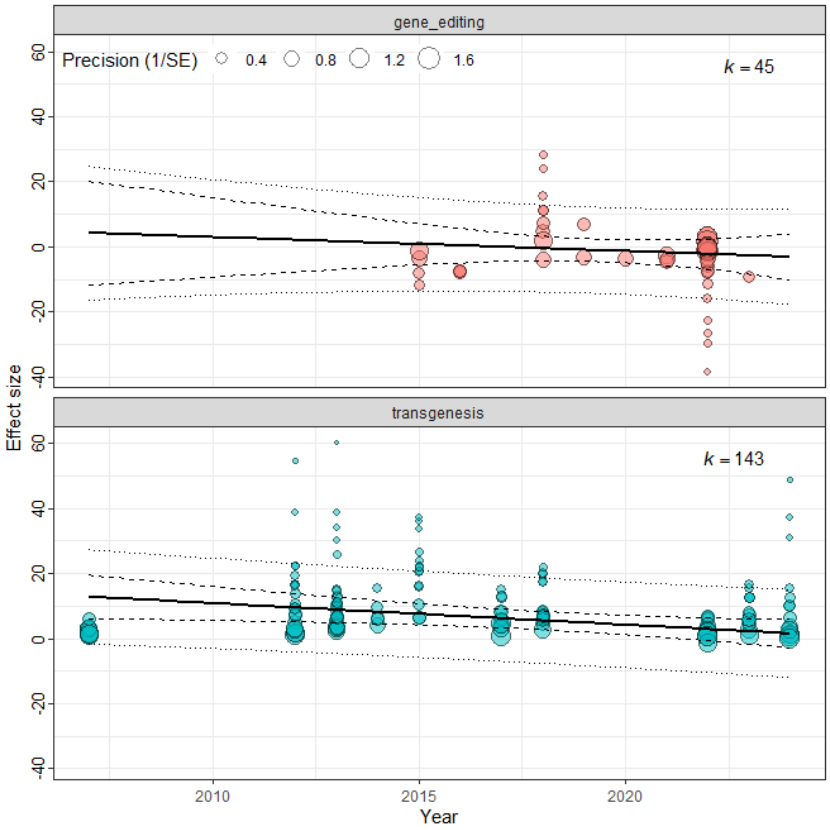
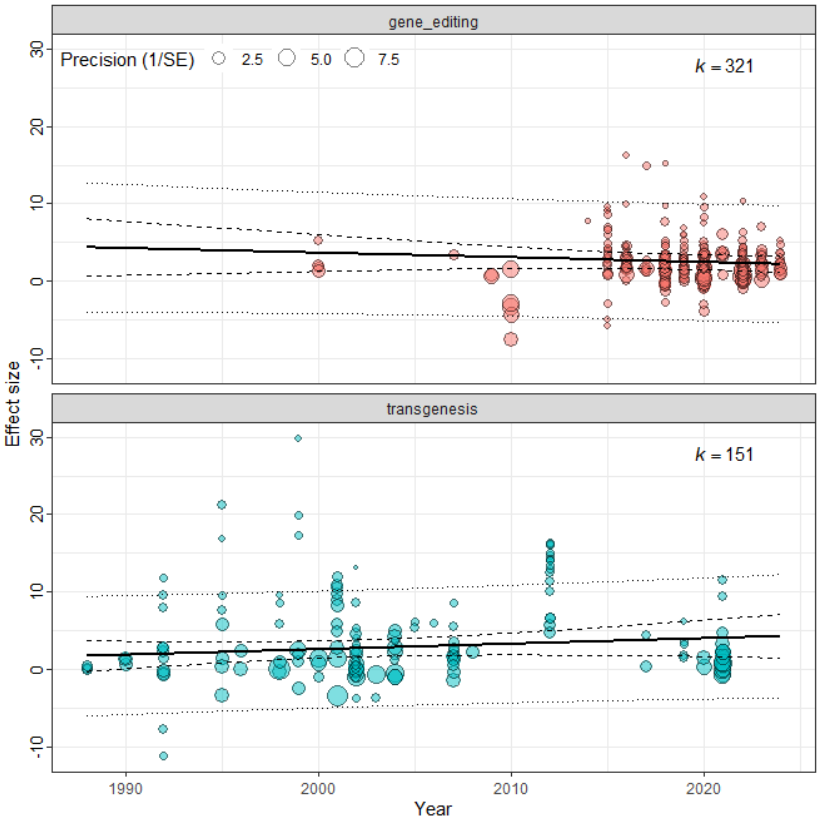


Fig. 10 Funnel plot of the standard error and the residuals of SMD from the full meta-regression model will all moderators included.

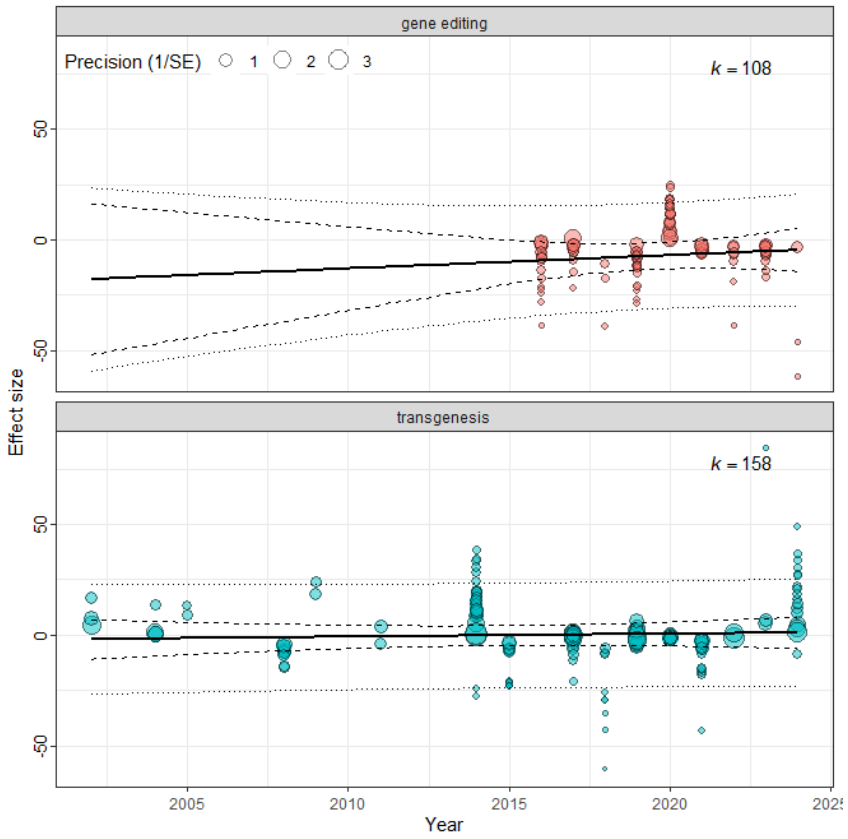
A


gene_editing
Precision (1/SE)
0.4
0.8
1.2
1.6
k = 45
transgenesis
k = 143
Effect size
Year
2010
2015
2020

B


gene_editing
Precision (1/SE)
2.5
5.0
7.5
k = 321
transgenesis
k = 151
Effect size
Year
1990
2000
2010
2020

C


gene editing
Precision (1/SE) 1 2 3
k = 108
transgenesis
k = 158
Effect size
Year
50
0
-50
2005
2010
2015
2020
2025

D

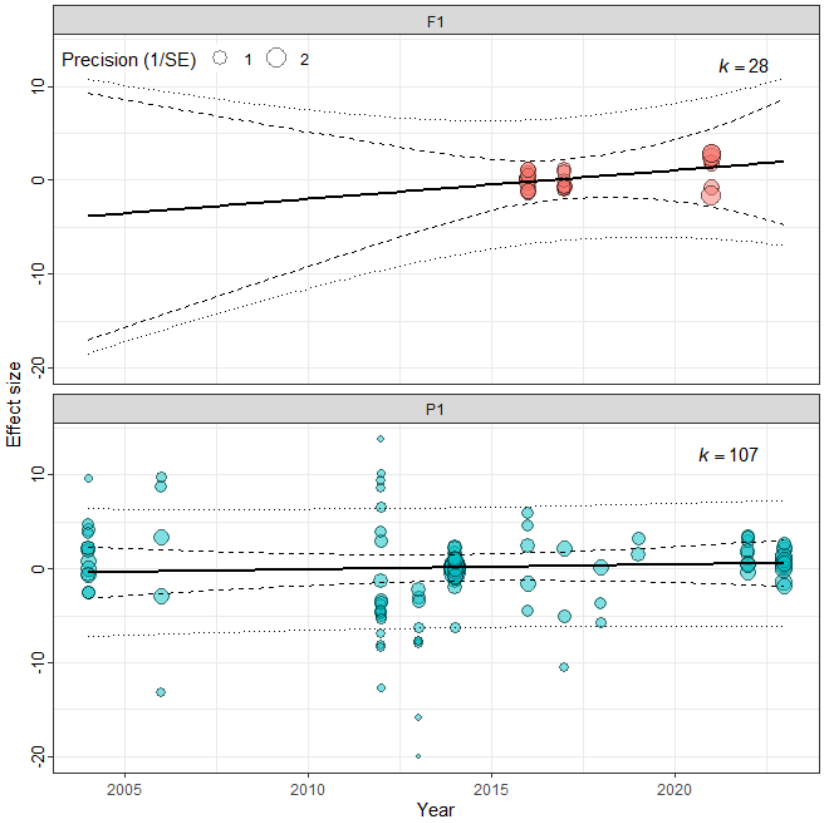

F1
Precision (1/SE) 1 2
k = 28
P1
k = 107
Effect size
Year
10
0
-10
-20
2005
2010
2015
2020

Fig. 11 Bubble plot of effect size plotted against centred publication year. (**A**) Gene/protein expression; (**B**) Growth; (**C**) Disease resistance; (**D**) Fatty acid

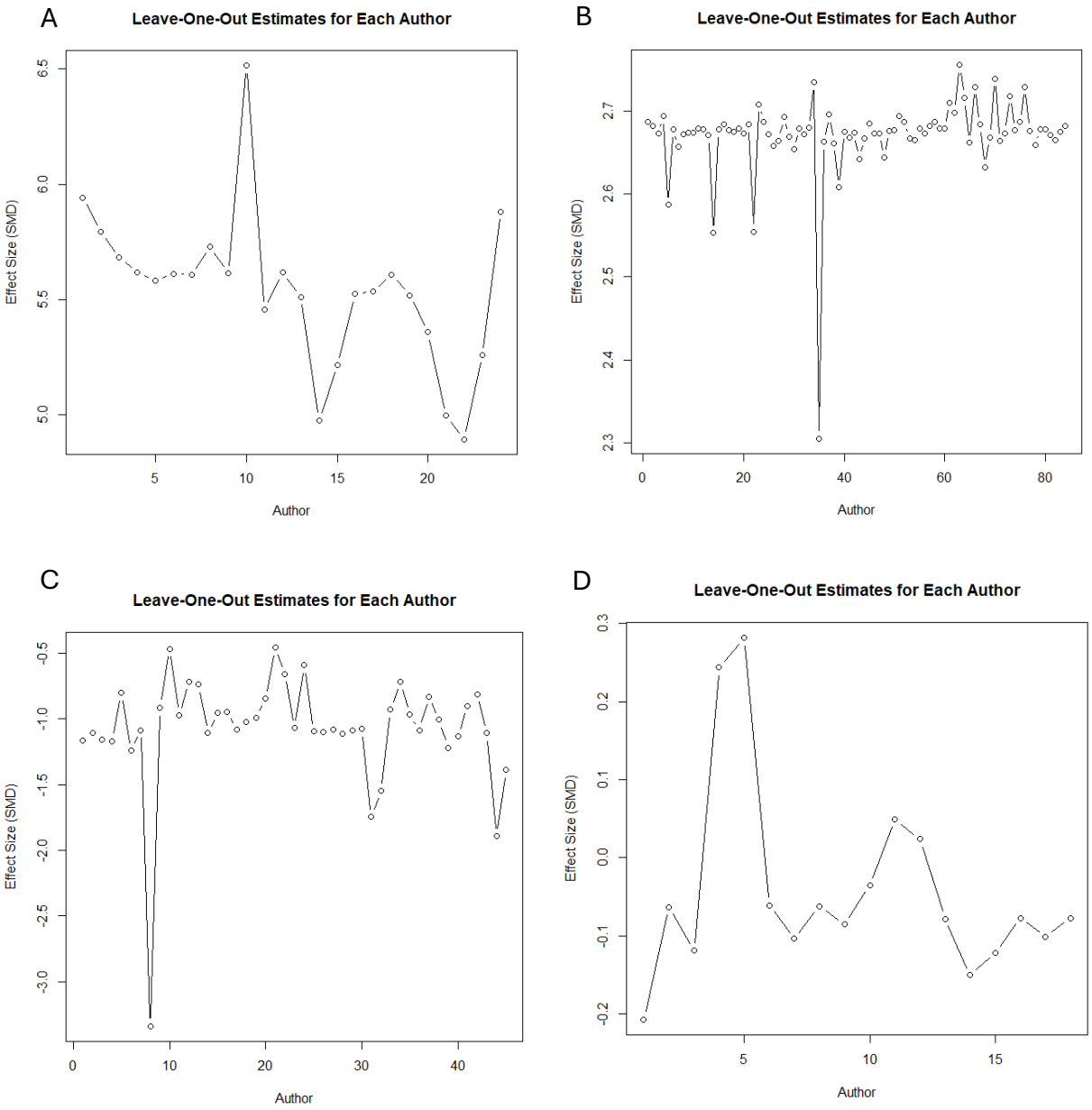


Fig. 12 Leave-one-out sensitivity analysis where one study was consecutively removed from the dataset: gene or protein expression (**A**), growth (**B**), disease resistance (**C**), and the content of fatty acid (**D**), and a new global meta-analytic mean was calculated. Studies are identified by first author name and publication year. Note: Outliers are indicated by red dots.

Supplementary Notes

Supplementary Note 1: Search string

We first conducted a scoping search on Google Scholar (16/09/2024) to screen the most relevant research papers using the keywords “Transgen* OR genome editing-animal”. Combined with our experience-collected review papers, we then built a word cloud to discern the most common words based on 50 papers (both research papers from Google Scholar and review papers from our empirical collection). We used the most frequently occurring relevant words from this word cloud to create keywords for our meta search string (Fig. 12).

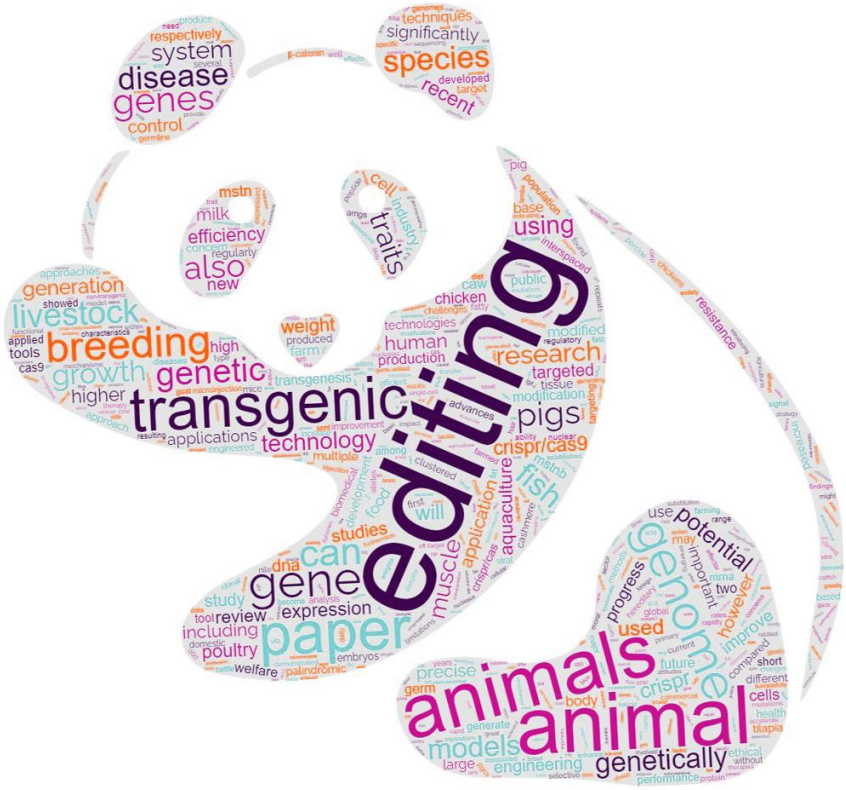


Fig. 12 The synthesized word cloud based on 50 selected titles/abstracts as search strings.

The table below (Table 3) shows the most frequent words from our scoping search, collected from 50 relevant titles/abstracts generated by a word cloud. Reference words used in our search string are highlighted in bold and italics. The words were chosen to be neither species specific nor biologically broad.

Table 3. The most frequent words from our scoping search collected from 50 relevant titles/abstracts generated through a word cloud.

Word	Frequency	Word	Frequency	Word	Frequency
editing	121	***disease***	29	chicken	14
genome	89	fish	28	food	14
gene	86	species	28	mstn	14
animal	72	***growth***	26	caw	13
animals	68	production	26	modified	13
genetic	61	genetically	25	body	12
transgenic	54	traits	25	farm	12
paper	53	modification	24	resistance	12
research	48	muscle	21	***transgenesis***	12
technology	42	poultry	17	Cas9	11
breeding	38	weight	17	***gene-edited***	11
CRISPR/Cas9	35	application	16	pig	11
aquaculture	33	improvement	16	performance	10
livestock	30	***engineering***	15	disease	10
pigs	30	improve	15	***fatty***	10

Distinct Boolean characters used for the search strings in each database (22/09/2024 − 25/09/2024).

Search string used for PubMed and Web of Science:

(“transgen*” OR “gene editing” OR “gene transfer” OR “CRISPR*”) AND (“trait” OR “growth” OR “disease” OR “body composition” OR “fatty acid”) AND (“animal” OR “livestock” OR “aquaculture”) NOT (“human” OR “monkey” OR “mice” OR “mouse” OR “rat” OR “zebrafish” OR “medaka”)

Search string used for SCOPUS:

TITLE-ABS-KEY (transgene) OR TITLE-ABS-KEY ("GENE TRANSFER") OR TITLE-ABS-KEY ("GENE EDITING") AND ((ALL (trait) OR ALL (growth) OR ALL (disease) OR ALL ("BODY COMPOSITION") OR ALL ("FATTY ACID")) AND (TITLE-ABS-KEY (animal) OR TITLE-ABS-KEY (livestock) OR TITLE-ABS-KEY (aquaculture))) AND NOT (TITLE-ABS-KEY (human) OR TITLE-ABS-KEY (monkey) OR TITLE-ABS-KEY (mice) OR TITLE-ABS-KEY (mouse) OR TITLE-ABS-KEY (rat) OR TITLE-ABS-KEY (zebrafish) OR TITLE-ABS-KEY (medaka)) AND (LIMIT-TO (DOCTYPE , "ar")) AND (LIMIT-TO (EXACTKEYWORD , "Article")) AND (LIMIT-TO (LANGUAGE , "English"))

Supplementary Note 2: Repeatability

Screening process

To ensure repeatability of the screening process, two analysts (Jinhai Wang and Lily Liu) screened titles/abstracts and full-texts independently and checked for agreement on included *vs*. excluded studies. Analyst 1 screened out all titles/abstracts to check the suitability of each study ($N = 10299$) while analyst 2 checked for repeatability by screening ~ 30% of titles/abstracts ($N = 3089$). Analysts 1 and 2 agreed on the inclusion or exclusion (i.e. suitability) of over 90% of the studies when screening abstracts. Analyst 1 then screened all full texts that passed the titles/abstracts screening stage ($N = 954$ studies) to determine whether a study was suitable to be included in the meta-dataset, while analyst 2 screened ~ 30% of full texts to test for repeatability ($N = 82$). Analysts 1 and 2 agreed on the inclusion or exclusion of over 95% of the studies when screening full texts.

Data extraction

Similarly, to assure repeatability of data extraction, two analysts checked data obtained from ~ 10% of papers (i.e. 15 studies and 62 effect sizes in *Gene/protein_expression* dataset; OR 15 studies and 73 effect sizes in *Growth* in dataset; OR 15 studies and 95 effect sizes in *Disease resistance* dataset; OR 15 studies and 134 effect sizes in *Fatty acid* dataset). To do this, analysts 1 and 2 first independently extracted data and calculated effect sizes (standardized mean difference, SMD) for each of the 73 rows of the meta-dataset. The difference of calculated SMDs from two analysts were not significant ($P > 0.05$), indicating strong repeatability.

Supplementary Note 3: Candidate species and outcomes

In our collected meta-dataset, species are restricted to animals, especially livestock (i.e. pig, cattle, goat or chicken), dog, rabbit, bird, and ray-finned fishes. The model animals, including monkey, mice/rat, zebrafish and medaka are out of our scope.

With respect to transgenesis, there is a diversity of promoters (i.e. CMV, SV40, β-actin or UBI) applied in recruited studies. Documents have recorded that gene/protein expression was affected by promoters; therefore, we considered the promoter as a moderator for our meta-analysis. Although different editing tools (i.e. ZFN, TALEN, or CRISPR/Cas9) and delivery methods (microinjection or electroporation) have been applied in different studies. It is not necessary to list editing tools or delivery methods as moderators, because the outcomes (gene/protein expression, growth, disease resistance and fatty acid) are not affected by editing tools or delivery approaches if the innate genes are successfully mutant with significantly reduced expression.

Four outcomes, including gene/protein expression, growth, disease resistance and fatty acid are major focused to compare the difference in trait performance between transgenic/gene-edited and wild type (WT) groups. For each outcome, we collected multiple parameters from a pool of published papers. For instance, gene/protein expressions mainly focused on the expression of targeted genes/proteins (transgene or target gene for editing and corresponding encoded proteins), and the expression of related genes/proteins are out of our scope. Two parameters, including mRNA and protein were extracted. Similarly, the parameters body weight, condition factor, FCE (feed conversion efficiency), myofiber number, myofiber size, plasma GH (growth hormone), SGR (specific growth rate), and weight gain belong to growth. Antibody response, CSR (cumulative survival rate), lysozyme activity, pathogen load, phagocytic activity, sign score, virus RNA and virus titer post pathogen infection were collected for disease resistance. In addition, the content of DHA (docosahexaenoic acid, 22:6n-3), EPA (eicosapentaenoic acid, 20:5n-3), ω-3 (omega-3), ω-6 and the ratio of ω-3/ω-6 were the main composition of fatty acid outcome. In this vein, we used multiple parameters for each outcome in our meta-analysis.

Supplementary Note 4: Trait performance and outcome definition

Trait performance, including gene/protein expression, growth, disease resistance and fatty acid are the main outcomes of the current meta-analysis. For all recruited studies, we collected data on traits of transgenic/gene-edited and WT groups as reported in the original papers. When a study reported one specific trait performance (gene/protein expression, growth, disease resistance or fatty acid) with multiple parameters, data on all parameters were collected/extracted. In addition, if a parameter (i.e. body weight) for the specific trait (i.e. growth) was recorded at different timepoints (i.e. sampling time), we also collected these data. Then a meta-regression analysis was applied to determine the effect of age on the target parameters. We created a broad category to describe different parameters of trait performance due to differences in terminology of parameters when describing a specific trait across studies. The categorization of these parameters is described below.

Trait	In meta-analysis	In original papers
Gene/protein expression	mRNA	mRNA abundance; the mRNA level; the gene expression level; relative gene expression level; relative transcriptional level; gene copy number; fold change
	protein	relative protein expression level; concentration of protein; the protein level; protein concentration
Growth	body weight	mean weight; average weight; carcass weight; wet weight; mean masses; live weight; birth weight; dressing percentage
	condition factor	condition factor; condition score
	FCE	feed conversion rate*; feed conversion efficiency
	myofiber number	fiber cell number; myofiber nuclei number; total number of fibers; percentage of fibers; myofiber density
	myofiber size	mean muscle fiber cross-sectional area; muscle fiber area; area of fiber cells; mean (total) area of fibers
	plasma GH	concentration of growth hormone
	SGR	specific growth rate; average growth speed; standard growth rate; percent of body weight increase
	weight gain	percentage weight gain; daily weight gain
Disease resistance	antibody response	antibody; ELIAS S/P ratio
	CSR	(cumulative) survival rate; mortality*
	lysozyme activity	lysozyme activity; polymerase activity
	pathogen load	number of virus-positive cells; colony-forming unit; viral growth; number of surviving bacteria; viral particle production
	phagocytic activity	phagocytic activity
	sign score	score for clinical signs; score for pathological changes
	virus RNA	relative expression of virus RNA; viral nucleic acid; fold change of viral RNA; viral RNA load level; viral RNA copies
	virus titer	Log_{10}TCID50; mean viral shedding titer; number of plaques
Fatty acid	DHA	docosahexaenoic acid; C22:6n-3
	EPA	eicosapentaenoic acid; C20:5n-3
	ω-3	omega-3; sum of n-3 polyunsaturated; total n-3 FA; n-3 PUFAs
	ω-6	omega-6; sum of n-6 polyunsaturated; total n-6 FA; n-6 PUFAs
	ω-6/ω-3	the ratio of omega-6/omega-3; n-6/n-3

Note: * FCE (feed conversion efficiency) = 1/feed conversion rate × 100%
CSR (cumulative survival rate) = 1 − mortality (%)

Supplementary Note 5: Calculation of effect size

Hedges'g served as the effect size because it corrects for differences in sampling effort among studies and adjusts for small sample sizes and was calculated using the following formula:

$$g = \frac{Mean_T - mean_C}{\sqrt{\frac{(n_1-1)SD_1^2+(n_2-2)SD_2^2}{n_1+n_2-2}}}$$

where $mean_T$ is the mean of the transgenic/gene-edited group, $mean_C$ is the mean of WT group, n_1 and n_2 are two sample sizes from these groups, and SD_1^2 and SD_2^2 are the estimated population variance of both groups.

Based on the formula above mentioned, we collected data on means, standard deviation/standard error (SD/SE), and sample sizes of each parameter in both groups from: text in the Methods/Results section, figures using ImageJ.JS (https://ij.imjoy.io/), the supplementary information or raw data provided with the paper, or a data repository, in that order. SD were determined using the formula $SD = SE*(n)^{1/2}$ if only SE and sample size (n) are supplied in a single publication. Additionally, when medians and inter-quartile ranges were reported, we converted these to SD.

Supplementary Note 6: Potential moderators in our meta-analysis models and their ranges/levels

Trait	Moderator	Ranges/Levels
Gene/protein expression	taxon	fish, pig, chicken, cattle, sheep, goat, rabbit, shrimp, bird, dog
	species/breed	channel catfish, common carp, Danish Landrace… (common name of species or breed from each category)
	method	transgenesis, gene editing
	gene	*IGF-I*, *mstn*, *cgGH*…(all target genes for transgenesis or gene editing)
	promoter	UBI, β-actin, CMV… (a promoter is displayed when the method is transgenesis)
	generation	P_1, F_1
	age	0 to months or years
	tissue	kidney, muscle, skin… (all tissues involved the detection of gene or protein expression)
Growth	taxon	fish, pig, chicken, cattle, sheep, goat, rabbit, shrimp, bird, dog
	species/breed	channel catfish, common carp, Danish Landrace… (common name of species or breed from each category)
	method	transgenesis, gene editing
	gene	*IGF-I*, *mstn*, *cgGH*…(all target genes for transgenesis or gene editing)
	promoter	UBI, β-actin, CMV… (a promoter is displayed when the method is transgenesis)
	generation	P_1, F_1
	age	0 to months or years
Disease resistance	taxon	fish, pig, chicken, cattle, sheep, goat, rabbit, shrimp, bird, dog
	species/breed	channel catfish, common carp, Danish Landrace… (common name of species or breed from each category)
	method	transgenesis, gene editing
	gene	*IGF-I*, *mstn*, *cgGH*…(all target genes for transgenesis or gene editing)
	promoter	UBI, β-actin, CMV… (a promoter is displayed when the method is transgenesis)
	generation	P_1, F_1
	pathogen	*Flavobacterium columnare*, *Edwardsiella ictaluri*, PERV… (all species of bacteria, virus or parasite used in a challenge experiment)
Fatty acid	taxon	fish, pig, chicken, cattle, sheep, goat, rabbit, shrimp, bird, dog
	species/breed	channel catfish, common carp, Danish Landrace… (common name of species or breed from each category)
	method	transgenesis, gene editing
	gene	*IGF-I*, *mstn*, *cgGH*…(all target genes for transgenesis or gene editing)
	promoter	UBI, β-actin, CMV… (a promoter is displayed when the method is transgenesis)
	generation	P_1, F_1
	age	0 to months or years
	source	*in vitro*, *in vivo* (kidney, liver, muscle…)

Supplementary Note 7: Recruited titles/abstracts for the word cloud construction

Paper 1: Research progress of gene editing technology CRISPR/Cas9 system in animal gene editing

Gene editing technology, from the beginning of RNA interference (RNAi) technology to efficiently developed enzyme technology, has been widely used in recent years. These efficient enzyme technologies include zinc finger nuclease (ZFN) technology, transcriptional activation-like effector nuclease (TALENs) technology, and clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated (Cas) 9 system (CRISPR/Cas9) technology. The CRISPR/Cas (Cas) system is a gene editing tool for DNA modification regulated by a short RNA and is a new type of genome editing tool that is faster, more efficient, and more accurate than the zinc finger nuclease and transcription activator-like effector nuclease. This article reviews the structure and function of the CRISPR/Cas system, and is aimed to outline the Cas9 design strategy, factors that affect the Cas9 gene editing efficiency, off-target detection and analysis methods, and especially the application in animal gene editing studies. Based on CRISPR/Cas9 gene editing has been successfully implemented in a variety of animals, and it is expected to become a new feasible way to establish animal models and study disease prevention in veterinary science and research.

Paper 2: The ethics of genome editing in non-human animals: a systematic review of reasons reported in the academic literature

In recent years, new genome editing technologies have emerged that can edit the genome of non-human animals with progressively increasing efficiency. Despite ongoing academic debate about the ethical implications of these technologies, no comprehensive overview of this debate exists. To address this gap in the literature, we conducted a systematic review of the reasons reported in the academic literature for and against the development and use of genome editing technologies in animals. Most included articles were written by academics from the biomedical or animal sciences. The reported reasons related to seven themes: human health, efficiency, risks and uncertainty, animal welfare, animal dignity, environmental considerations and public acceptability. Our findings illuminate several key considerations about the academic debate, including a low disciplinary diversity in the contributing academics, a scarcity of systematic comparisons of potential consequences of using these technologies, an underrepresentation of animal interests, and a disjunction between the public and academic debate on this topic. As such, this article can be considered a call for a broad range of academics to get increasingly involved in the discussion about genome editing, to incorporate animal interests and systematic comparisons, and to further discuss the aims and methods of public involvement.

Paper 3: Genome editing strategies to protect livestock from viral infections

The livestock industry is constantly threatened by viral disease outbreaks, including infections with zoonotic potential. While preventive vaccination is frequently applied, disease control and eradication also depend on strict biosecurity measures. Clustered regularly interspaced palindromic repeats (CRISPR) and associated proteins (Cas) have been repurposed as genome

editors to induce targeted double-strand breaks at almost any location in the genome. Thus, CRISPR/Cas genome editors can also be utilized to generate disease-resistant or resilient livestock, develop vaccines, and further understand virus–host interactions. Genes of interest in animals and viruses can be targeted to understand their functions during infection. Furthermore, transgenic animals expressing CRISPR/Cas can be generated to target the viral genome upon infection. Genetically modified livestock can thereby reduce disease outbreaks and decrease zoonotic threats.

Paper 4: Germline modification of domestic animals

Genetically-modified domestic animal models are of increasing significance in biomedical research and agriculture. As authentic ES cells derived from domestic animals are not yet available, the prevailing approaches for engineering genetic modifications in those animals are pronuclear microinjection and somatic cell nuclear transfer (SCNT, also known as cloning). Both pronuclear microinjection and SCNT are inefficient, costly, and time-consuming. In animals produced by pronuclear microinjection, the exogenous transgene is usually inserted randomly into the genome, which results in highly variable expression patterns and levels in different founders. Therefore, significant efforts are required to generate and screen multiple founders to obtain animals with optimal transgene expression. For SCNT, specific genetic modifications (both gain-of-function and loss-of-function) can be engineered and carefully selected in the somatic cell nucleus before nuclear transfer. SCNT has been used to generate a variety of genetically modified animals such as goats, pigs, sheep and cattle; however, animals resulting from SCNT frequently suffer from developmental abnormalities associated with incomplete nuclear reprogramming. Other strategies to generate genetically-modified animals rely on the use of the spermatozoon as a natural vector to introduce genetic material into the female gamete. This sperm mediated DNA transfer (SMGT) combined with intracytoplasmatic sperm injection (ICSI) has relatively high efficiency and allows the insertion of large DNA fragments, which, in turn, enhance proper gene expression. An approach currently being developed to complement SCNT for producing genetically modified animals is germ cell transplantation using genetically modified male germline stem cells (GSCs). This approach relies on the ability of GSCs that are genetically modified in vitro to colonize the recipient testis and produce donor derived sperm upon transplantation. As the genetic change is introduced into the male germ line just before the onset of spermatogenesis, the time required for the production of genetically modified sperm is significantly shorter using germ cell transplantation compared to cloning or embryonic stem (ES) cell based technology. Moreover, the GSC-mediated germline modification circumvents problems associated with embryo manipulation and nuclear reprogramming. Currently, engineering targeted mutations in domestic animals using GSCs remains a challenge as GSCs from those animals are difficult to maintain in vitro for an extended period of time. Recent advances in genome editing techniques such as Zinc-Finger Nucleases (ZFNs), Transcription Activator-like Effector Nucleases (TALENs) and Clustered Regularly Interspaced Short Palindromic Repeats (CRISPRs) greatly enhance the efficiency of engineering targeted genetic change in domestic animals as demonstrated by the generation of several gene knock-out pig and cattle models using those techniques. The potential of GSC-

mediated germline modification in making targeted genetic modifications in domestic animal models will be maximized if those genome editing techniques can be applied in GSCs.

Paper 5: Evaluation and detection of genome-edited livestock

With the development of genome-editing technology, the process of genetic improvement of livestock and poultry breeds has been greatly accelerated, and a large number of genetically engineered animals with excellent traits have been developed. These are also known as genome-edited animals. The emergence of these genetically engineered animals makes people realize that they have great application value for the shortage of livestock resources, the protection of the ecological environment, and the development of medical technology. However, the biosafety problems that might be caused by the introduction of foreign genes into the genome are also deeply concerned. In order to avoid potential biosafety problems, the evaluation and detection of genetically engineered animals are widely used. In this chapter, the biosafety evaluation principles of genetically engineered animals and the commonly used detection technologies for genetically engineered animals are elaborated in detail, with a view to providing references for the safe production of genetically engineered animals.

Paper 6: Application of CRISPR/Cas9 System in Establishing Large Animal Models

The foundation for investigating the mechanisms of human diseases is the establishment of animal models, which are also widely used in agricultural industry, pharmaceutical applications, and clinical research. However, small animals such as rodents, which have been extensively used to create disease models, do not often fully mimic the key pathological changes and/or important symptoms of human disease. As a result, there is an emerging need to establish suitable large animal models that can recapitulate important phenotypes of human diseases for investigating pathogenesis and developing effective therapeutics. However, traditional genetic modification technologies used in establishing small animal models are difficultly applied for generating large animal models of human diseases. This difficulty has been overcome to a great extent by the recent development of gene editing technology, especially the clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9). In this review, we focus on the applications of CRISPR/Cas9 system to establishment of large animal models, including nonhuman primates, pigs, sheep, goats and dogs, for investigating disease pathogenesis and treatment. We also discuss the limitations of large animal models and possible solutions according to our current knowledge. Finally, we sum up the applications of the novel genome editing tool Base Editors (BEs) and its great potential for gene editing in large animals.

Paper 7: Mapping and editing animal mitochondrial genomes: can we overcome the challenges?

The animal mitochondrial genome, although small, can have a big impact on health and disease. Non-pathogenic sequence variation among mitochondrial DNA (mtDNA) haplotypes influences traits including fertility, healthspan and lifespan, whereas pathogenic mutations are linked to incurable mitochondrial diseases and other complex conditions like ageing, diabetes, cancer and neurodegeneration. However, we know very little about how mtDNA genetic variation contributes

to phenotypic differences. Infrequent recombination, the multicopy nature and nucleic acid-impenetrable membranes present significant challenges that hamper our ability to precisely map mtDNA variants responsible for traits, and to genetically modify mtDNA so that we can isolate specific mutants and characterize their biochemical and physiological consequences. Here, we summarize the past struggles and efforts in developing systems to map and edit mtDNA. We also assess the future of performing forward and reverse genetic studies on animal mitochondrial genomes.

Paper 8: Predicting Public Attitudes Toward Gene Editing of Germlines: The Impact of Moral and Hereditary Concern in Human and Animal Applications

Background and Objective: New and more efficient methods of gene editing have intensified the ethical and legal issues associated with editing germlines. Yet no research has separated the impact of hereditary concern on public attitudes from moral concern. This research compares the impact these two concerns have on public attitudes across five applications including, the prevention of human disease, human and animal research, animals for the use of human food and the enhancement of human appearance.

Methods: A sample of 1004 Australians responded to either a telephone (n = 501; randomly selected) or online survey (n = 503; sourced by Qualtrics). Both samples were representative in terms of States and Territories as well as gender (51% female), though the online sample was younger (M = 40.64, SD = 16.98; Range = 18–87) than the telephone sample (M = 54.79, SD = 18.13; Range = 18–96). A 5 (application) by 3 (type of cell) within groups design was utilized, where all respondents reported their level of approval with scientists editing genes across the 15 different contexts. Multilevel modeling was used to examine the impact of moral (embryo vs. germ) and hereditary (germ vs. somatic) concern on attitudes across all applications.

Results: Australians were comfortable with editing human and animal embryos, but only for research purposes and to enhance human health. The effect of moral concern was stronger than hereditary concern, existing in all applications except for the use of animals for human purposes. Hereditary concern was only found to influence attitudes in two applications: improving human health and human research. Moral concern was found to be accentuated amongst, women, more religious individuals and those identifying as Australian, while hereditary concern was strongest amongst non-Australians, those with stronger trust in scientists, and more religious respondents.

Conclusion: Moral and hereditary concerns are distinct, and require different approaches to public education, engagement and possibly regulation. Further research needs to explore hereditary concern in relation to non-human applications, and the reasons underlying cultural and gender differences.

Paper 9: *In vivo* genome editing thrives with diversified CRISPR technologies

Prokaryotic type II adaptive immune systems have been developed into the versatile CRISPR technology, which has been widely applied in site-specific genome editing and has revolutionized biomedical research due to its superior efficiency and flexibility. Recent studies have greatly

diversified CRISPR technologies by coupling it with various DNA repair mechanisms and targeting strategies. These new advances have significantly expanded the generation of genetically modified animal models, either by including species in which targeted genetic modification could not be achieved previously, or through introducing complex genetic modifications that take multiple steps and cost years to achieve using traditional methods. Herein, we review the recent developments and applications of CRISPR-based technology in generating various animal models, and discuss the everlasting impact of this new progress on biomedical research.

Paper 10: Advantages and Challenges of CRISPR-Cas9 Applications in Animal Modeling: A Concise Review

CRISPR is an extraordinarily powerful technique regulating any target gene across the genome with promising therapy intentions. CRISPR-Cas9 is a convenient tool for gene manipulation. Notwithstanding this, the broad consequence of human gene editing, particularly germinal genes, cannot be predicted. Firstly, once edited, the genes would be part of the human population for successive generations and may be impossible to remove from humanity; secondly, success is not guaranteed; thirdly, the fidelity of editing, as it could affect unrelated genes or unspecified segments of DNA; and last but not least, its influence on gene interaction, network, and signaling pathways could be difficult to be predicted. CRISPR-Cas9 mostly includes precise genome editing, rapidity and cost-effectiveness, creation of disease models, study of gene function, applications in gene therapy and translation research and wide diversity for species. The technique also ignited the moral and ethical concerns of scientific community. Ethics and safety approval for gene modification in human cells is required by the National Institutes of Health (NIH). The NIH does not currently fund studies of CRISPR in human embryos and opposes the CRISPR utilization in germline cells because these alterations would be permanent and heritable. The technology has promised with the most profound implications for cancer therapy. Recent advances in CRISPR-based technology is redefining how cancer is studied and potentially improves anti-cancer therapies. One way to improve the technology is to use machine-learning approaches to comprehending CRISPR errors and predicting more specific edits and repairing outcomes.

Paper 11: Gene editing in monogenic autism spectrum disorder: animal models and gene therapies

Autism spectrum disorder (ASD) is a lifelong neurodevelopmental disease, and its diagnosis is dependent on behavioral manifestation, such as impaired reciprocal social interactions, stereotyped repetitive behaviors, as well as restricted interests. However, ASD etiology has eluded researchers to date. In the past decades, based on strong genetic evidence including mutations in a single gene, gene editing technology has become an essential tool for exploring the pathogenetic mechanisms of ASD via constructing genetically modified animal models which validates the casual relationship between genetic risk factors and the development of ASD, thus contributing to developing ideal candidates for gene therapies. The present review discusses the progress in gene editing techniques and genetic research, animal models established by gene editing, as well as gene therapies in ASD. Future research should focus on improving the validity of animal models, and

reliable DNA diagnostics and accurate prediction of the functional effects of the mutation will likely be equally crucial for the safe application of gene therapies.

Paper 12: Base editing and prime editing in laboratory animals

Genome editing by programmable RNA-dependent Cas endonucleases has revolutionised the field of genome engineering, achieving targeted genomic change at unprecedented efficiencies with considerable application in laboratory animal research. Despite its ease of use and wide application, there remain concerns about the precision of this technology and a number of unpredictable consequences have been reported, mostly resulting from the DNA double-strand break (DSB) that conventional CRISPR editing induces. In order to improve editing precision, several iterations of the technology been developed over the years. Base editing is one of most successful developments, allowing for single base conversions but without the need for a DSB. Cytosine and adenine base editing are now established as reliable methods to achieve precise genome editing in animal research studies. Both cytosine and adenine base editors have been applied successfully to the editing of zygotes, resulting in the generation of animal models. Similarly, both base editors have achieved precise editing of point mutations in somatic cells, facilitating the development of gene therapy approaches. Despite rapid progress in optimising these tools, base editing can address only a subset of possible base conversions within a relatively narrow window and larger genomic manipulations are not possible. The recent development of prime editing, originally defined as a simple 'search and replace' editing tool, may help address these limitations and could widen the range of genome manipulations possible. Preliminary reports of prime editing in animals are being published, and this new technology may allow significant advancements for laboratory animal research.

Paper 13: Gene Editing, Animal Disenhancement and Ethical Debates: A Conundrum for Business Ethics?

Despite the potential of genetic disenhancement to create livestock incapable of pain and thus reduce animal suffering in industrial farming, ethical theorists have rejected disenhancement as intuitively unethical or as part of a broader dismissal of industrial farming. Although criticisms of industrial farming may be valid, the suffering of animals involved still needs to be addressed, and business ethics is specially placed to do so. In this chapter, a brief overview of the related ethical issues of industrial farming and disenhancement are outlined, and practical steps businesses should make to address animal suffering are provided. Explicit Corporate Social Responsibility policies that reflect the interest of animals, workers and consumers as stakeholders should be put in place, which would provide a mechanism to make businesses accountable for genetic modification and animal welfare more generally.

Paper 14: P7002 Skin-specific transgenic expression of ovine β-catenin in mice

β-catenin is an evolutionarily conserved molecule that functions as a crucial effector in the development of hair follicles. The primary goal of the present study was to investigate the effect of ovine β-catenin on the hair follicle. Transgenic mice were produced by pronuclear microinjection with skin specific promoter of human keratin14 (k14). Transgenic mice were

identified by PCR and Southern blot analysis and the β-catenin gene could be stably inherited was validated. Furthermore, qRT-PCR and Western blot analysis indicated that high level of β-catenin was expressed specifically in the skin tissue. The β-catenin was visualized strongly expressed in the inner root sheath and dermal papilla of the hair follicle based on immunohistochemical analysis. Also, the results of the frozen sections analysis showed different phenotypes in follicle position of transgenic individuals relative to their negative full-sibs or wild mice, that every follicle contained two or three hairs in 70% of the random sites of the transgenic skin sections, other than one hair in wild mice. These results suggested that the transgenic mice model with overexpression of ovine β-catenin was produced successfully, provides a material for deciphering the molecular mechanism involved in hair follicle growth and development, and provides a foundation for further producing β-catenin overexpression in transgenic sheep.

Paper 15: Genome Editing Strategies to Protect Livestock from Viral Infections

The livestock industry is constantly threatened by viral disease outbreaks, including infections with zoonotic potential. While preventive vaccination is frequently applied, disease control and eradication also depend on strict biosecurity measures. Clustered regularly interspaced palindromic repeats (CRISPR) and associated proteins (Cas) have been repurposed as genome editors to induce targeted double-strand breaks at almost any location in the genome. Thus, CRISPR/Cas genome editors can also be utilized to generate disease-resistant or resilient livestock, develop vac-cines, and further understand virus–host interactions. Genes of interest in animals and viruses can be targeted to understand their functions during infection. Furthermore, transgenic animals ex- pressing CRISPR/Cas can be generated to target the viral genome upon infection. Genetically modified livestock can thereby reduce disease outbreaks and decrease zoonotic threats.

Paper 16: Crispr/Cas9 and its current application status on pig breeding

Pork provides substantial portion of daily meals for human. To meet the global demand for growing population, implementation of advanced technologies in pig breeding is required. The most recently gene editing CRISPR/Cas9 system provides novel tools to pig breeders to improve animal welfare, productivity, and performance. This paper aims to give principles of genome editing techniques and the latest update on CRISPR/Cas9 application on pig industry. We will also review the possible choices for delivering CRISPR/Cas9 system in pig research. It is hoped that with the adaptation of CRISPR/Cas9 technology, rapid genetic understanding and utilization in pig breeding will soon accelerate.

Paper 17: Is Animal Welfare Promoting Hornless Cattle? Assessing Consumer's Valuation for Milk from Gene-edited Cows under Different Information Regimes

Due to its ability to achieve genetic engineering goals without transgene modifications, gene-editing is fast becoming a predominant genetic-engineering breeding technique and a range of food-related applications have already been developed. Yet, it remains unclear whether consumers would perceive gene-edited food products differently from so-called first-generation genetic engineering or from genetic modification, and how information about its benefits might alter consumer choice. Focusing on a recent gene-editing application in animal production, the genetic

dehorning of dairy cows, this study uses data from a survey of 1,000 US consumers to determine: (i) consumer willingness-to-pay for milk from cows that have been gene-edited to be hornless; and (ii) consumers' response to information about how the gene-editing technology works, how it differs from genetic modification, and its benefits for animal welfare. Information treatments utilise videos to maximise respondents' understanding. Results from parametric and semi-parametric choice models suggest that: (i) information on animal welfare has the strongest effect on consumer willingness-to-pay for milk produced from conventionally and genetically dehorned cows; and (ii) that providing respondents with more information leads to an increasingly wider spread of the preference distributions in the positive and negative domain uncovering a further polarisation of preferences and confirming the heterogeneous impact of information on preferences.

Paper 18: Genome editing in avian research- an update review

Chicken contributes significant portion in human daily meals and serves as a research model in genome editing. As a result, research in elucidating gene functions and directional modification of the phenotypes aimed for customerdriven needs are highly emphasized in broiler industry. The development of genome editing technologies including zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) and the latest cluster regularly interspaced short palindromic repeat (CRISPR)/Cas9 (CRISPR/Cas9) revolutionized the understandings in chicken development and offered novel tools in generating genome edited chicken to improve chicken productivity and health. This review will provide the principles of genome editing techniques and latest updates of their applications in improving chicken productivity and health.

Paper 19: One-step *in vivo* gene knock-out in porcine embryos using recombinant adeno-associated viruses

Introduction: Gene-edited pigs have become prominent models for studying human disease mechanisms, gene therapy, and xenotransplantation. CRISPR (clustered regularly interspaced short palindromic repeats)/CRISPR-associated 9 (CRISPR/Cas9) technology is a widely employed tool for generating gene-edited pigs. Nevertheless, delivering CRISPR/Cas9 to pre-implantation embryos has traditionally posed challenges due to its reliance on intricate micromanipulation equipment and specialized techniques, resulting in high costs and time-consuming procedures. This study aims to introduce a novel one-step approach for generating genetically modified pigs by transducing CRISPR/Cas9 components into pre-implantation porcine embryos through oviductal injection of recombinant adeno-associated viruses (rAAV).

Methods: We first used rAAV-1, rAAV-6, rAAV-8, rAAV-9 expressing EGFP to screen for rAAV serotypes that efficiently target porcine embryos, and then, to achieve efficient expression of CRISPR/Cas9 *in vivo* for a short period, we packaged sgRNAs targeting the GHR genes to self-complementary adeno-associated virus (scAAV), and Cas9 proteins to single-stranded adeno-associated virus (ssAAV). The efficiency of porcine embryos -based editing was then validated *in vitro*. The feasibility of this one-step method to produce gene-edited pigs using rAAV-CRISPR/Cas9 oviductal injection into sows within 24 h of conception was then validated.

Results: Our research firstly establishes the efficient delivery of CRISPR/Cas9 to pig zygotes, both *in vivo* and *in vitro*, using rAAV6. Successful gene editing in pigs was achieved through oviductal injection of rAAV-CRISPR/Cas9.

Conclusion: This method circumvents the intricate procedures involved in *in vitro* embryo manipulation and embryo transfers, providing a straightforward and cost-effective approach for the production of gene-edited pigs.

Paper 19: Efficient base editing for multiple genes and loci in pigs using base editors

Cytosine base editors (CBEs) enable programmable C-to-T conversion without DNA double-stranded breaks and homology-directed repair in a variety of organisms, which exhibit great potential for agricultural and biomedical applications. However, all reported cases only involved C-to-T substitution at a single targeted genomic site. Whether C-to-T substitution is effective in multiple sites/loci has not been verified in large animals. Here, by using pigs, an important animal for agriculture and biomedicine, as the subjective animal, we showed that CBEs could efficiently induce C-to-T conversions at multiple sites/loci with the combination of three genes, including DMD, TYR, and LMNA, or RAG1, RAG2, and IL2RG, simultaneously, at the embryonic and cellular levels. CBEs also could disrupt genes (pol gene of porcine endogenous retrovirus) with dozens of copies by introducing multiple premature stop codons. With the CBEs, pigs carrying single gene or multiple gene point mutations were generated through embryo injection or nuclear transfer approach.

Paper 20: Genetically Modifying Livestock for Improved Welfare: A Path Forward

In recent years, humans' ability to selectively modify genes has increased dramatically as a result of the development of new, more efficient, and easier genetic modification technology. In this paper, we argue in favor of using this technology to improve the welfare of agricultural animals. We first argue that using animals genetically modified for improved welfare is preferable to the current status quo. Nevertheless, the strongest argument against pursuing gene editing for welfare is that there are alternative approaches to addressing some of the challenges of modern agriculture that may offer ethical advantages over genetic modification; namely, a dramatic shift towards plant-based diets or the development of in vitro meat. Nevertheless, we provide reasons for thinking that despite these possible comparative disadvantages there are important reasons for continuing the pursuit of welfare improvements via genetic modification.

Paper 21: Production of hypoallergenic milk from DNA-free beta-lactoglobulin (BLG) gene knockout cow using zinc-finger nucleases mRNA

The whey protein β-lactoglobulin (BLG) is a major milk allergen which is absent in human milk. Here, we for the first time generated DNA-free BLG bi-allelic knockout cow by zinc-finger nuclease (ZFNs) mRNA and produced BLG-free milk. According to the allergenicity evaluation of BLG-free milk, we found it can trigger lower allergic reaction of Balb/c mice including the rectal temperature drop and the allergen-specific immunoglobulin IgE production; BLG free-milk was easily digested by pepsin at 2 min, while BLG in control milk was still not completely digested

after 60 min, and the binding of IgE from cow's milk allergy (CMA) patients to BLG free-milk was significantly lower than that to the control milk. Meanwhile, the genome sequencing revealed that our animal is free of off-target events. Importantly, editing animal genomes without introducing foreign DNA into cells may alleviate regulatory concerns related to foods produced by genome edited animals. Finally, the ZFNs-mediated targeting in cow could be transmitted through the germline by breeding. These findings will open up unlimited possibilities of modifying milk composition to make it more suitable for human health and also improve the functional properties of milk.

Paper 22: Generation of a genetically modified pig model with CREBRF^{R457Q} variant

Obesity is among the strongest risk factors for type 2 diabetes (T2D). The CREBRF missense allele rs373863828 (p. Arg457Gln, p. R457Q) is associated with increased body mass index but reduced risk of T2D in people of Pacific ancestry. To investigate the functional consequences of the CREBRF variant, we introduced the corresponding human mutation R457Q into the porcine genome. The CREBRFR457Q pigs displayed dramatically increased fat deposition, which was mainly distributed in subcutaneous adipose tissue other than visceral adipose tissue. The CREBRFR457Q variant promoted preadipocyte differentiation. The increased differentiation capacity of precursor adipocytes conferred pigs the unique histological phenotype that adipocytes had a smaller size but a greater number in subcutaneous adipose tissue (SAT) of CREBRFR457Q variant pigs. In addition, in SAT of CREBRFR457Q pigs, the contents of the peroxidative metabolites 4-hydroxy-nonenal and malondialdehyde were significantly decreased, while the activity of antioxidant enzymes, such as glutathione peroxidase, superoxide dismutase, and catalase, was increased, which was in accordance with the declined level of the reactive oxygen species (ROS) in CREBRFR457Q pigs. Together, these data supported a causal role of the CREBRFR457Q variant in the pathogenesis of obesity, partly via adipocyte hyperplasia, and further suggested that reduced oxidative stress in adipose tissue may mediate the relative metabolic protection afforded by this variant despite the related obesity.

Paper 23: Generation of VEGF knock-in Cashmere goat via the CRISPR/Cas9 system

Cashmere is a rare and specialised animal fibre, which grows on the outer skin of goats. Owing its low yield and soft, light, and warm properties, it has a high economic value. Here, we attempted to improve existing cashmere goat breeds by simultaneously increasing their fibre length and cashmere yield. We attempted this by knocking in the vascular endothelial growth factor (*VEGF*) at the fibroblast growth factor 5(*FGF5*) site using a gene editing technology and then studying its hair growth-promoting mechanisms. We show that a combination of RS-1 and NU7441 significantly improve the efficiency of CRISPR/Cas9-mediated, homologous-directed repair without affecting the embryo cleavage rate or the percentages of embryos at different stages. In addition, we obtained a cashmere goat, which integrated the *VEGF* gene at the *FGF5* site, and the cashmere yield and fibre length of this gene-edited goat were improved. Through next-generation sequencing, we found that the up-regulation of *VEGF* and the down-regulation of *FGF5* affected the cell cycle, proliferation, and vascular tone through the PI3K-AKT signalling pathway and at

extracellular matrix-receptor interactions. Owing to this, the gene-edited cashmere goat showed impressive cashmere performance. Overall, in this study, we generated a gene-edited cashmere goat by integrating *VEGF* at the *FGF5* site and provided an animal model for follow-up research on hair growth mechanisms.

Paper 24: Genome Editing and Farmed Animal Breeding: Ethical and Legal Considerations

This dissertation was written as part of the MSc in Bioeconomy: Biotechnology and Law at the University Centre of International Programmes of Studies of the International Hellenic University, in Thessaloniki, Greece. The emergence of innovative tools in biotechnology together with a sustainable and ethical food supply chain can contribute to the complex challenges in the food and farming sector, where there is an urgent call for more protein-based food. Genome editing (GE) can significantly improve the speed of meeting increased productivity needs in livestock breeding. Purpose – This work aims to identify and examine ethical and l50egal questions in GE farmed animals and its rationale is attributed to the paucity of research in this research area, given the recent developments in biological research and biotechnology. Originality – This work is, at the moment, among limited international literature on the subject and among the first works in Greece. Methodology – The integrative review is considered suitable for this dissertation, which examines a newly emerging topic. The purpose is to critically synthesise research findings and creatively combine perspectives from different research fields. Discussion and Limitations – Limitations included the lack of previous studies in this field, the broad formation of research objectives and focus of research on already existing and not primary data. The research clearly illustrates that the global food and farming system currently operates unsustainably and in a morally indispensable way. Conclusions and Future Research Recommendations – A multidisciplinary approach will reinforce the proactive assessment of the novel technologies and their socio-ethical implications. On this basis, it is recommended that future research focuses on a comprehensive regulatory framework with appropriate bioethics standards that reflects public perceptions and integrates essential socio-economic factors. Keywords: genome editing; farmed animal breeding; ethics; law; public perceptions.

Paper 25: Growth performance and meat characteristics of Awassi sheep that holds the Callipyge gene

For improving meat quality and quantity of Awassi sheep, frozen semen of four Rambouillit rams (R, homozygous for the mutation of the Callipyge gene, CLPG) was imported from the United States (Utah University). The introgression of the CLPG into Awassi was started by producing the F1 by crossing R with Awassi (AW) ewes, while the first backcross (FBC; 75% AW and 25% R) was formed by using the F1 Callipyge carriers with AW ewes. To examine the performance of the FBC (heterozygous for CLPG) compared with the AW, a fattening trial was designed using 16 weaned male lambs (eight from each of AW and FBC lambs) kept in individual pens for 98 d. A well-balanced ration was offered, feed intake was recorded on a daily basis, and weights were monitored weekly. At the end of the fattening period, lambs were slaughtered to investigate the carcass cuts and meat characteristics. Callipyge carrier lambs (CAW) exceeded AW lambs in their

slaughter weight ($P = 0.0002$) and the average daily gain for AW and CAW were 0.189 and 0.332 kg/d, respectively ($P < 0.0001$). Awassi lambs consumed 1.217 kg feed/kg live weight more than CAW ($P = 0.0254$). The slaughter weight of the CAW was higher than the AW ($P = 0.0001$); CAW weighed 50.9 kg at slaughter age (176.5 ± 1.34 d), while AW weighed 37.07 kg. The hot and cold carcass weights, dressing percentage, and shoulders, legs, rack, and loin weights were significantly higher in the CAW. Fat tail weight was nonsignificantly affected by the genotype. The heart, liver, kidney, and kidney fat weights were significantly higher in CAW. Longissimus, total leg muscle weight, intermuscular and subcutaneous fat, and total bone weights were higher in CAW compared with AW. The ratio of total muscle to leg weight and total bone to leg weight were higher in CAW, while the intermuscular fat/leg weight subcutaneous fat/leg weight were nonsignificantly affected by the genotype. Eye muscle area was 14.8099 in AW, while 25.3521 ± 1.4086 in CAW ($P < 0.0001$). CAW was higher than AW of about 0.168 kg in the eye muscle weight ($P = 0.0003$). Shearforce values were 7.2844 in CAW compared with 3.2187 in AW ($P < 0.0002$). Meat color nonsignificantly differs between CAW and AW. CLPG can be used for improving meat quantity and quality of AW.

Paper 26: Heritable gene disruption in goats with CRISPR/Cas9 results in expected phenotypes

Precision genetic engineering will accelerate the genetic improvement of livestock for agriculture and biomedicine. Recent advances in the study of the CRISPR/Cas9 system have provided a precise and versatile approach for genome editing in various species. However, the applicability and efficiency of this method in large animal models, such as the goat, have not been extensively studied. Here, we successfully generated gene-modified goats with either one or both genes disrupted through co-injection of onecell-stage embryos with Cas9 mRNA and sgRNAs targeting two genes (MSTN and FGF5) with well-known function. The targeting efficiency of MSTN and FGF5 in cultured primary fibroblasts was as high as 60%, while the efficiency of disrupting MSTN and FGF5 in 98 tested animals was 15 and 21%, respectively, and targeting two genes simultaneously was 10%. The on- and off-target mutations of the target genes in fibroblasts, as well as in somatic tissues and testis of founder animals, were carefully analyzed, indicating that simultaneous editing of several sites can be achieved in large animals. We further show that the utility of the CRISPR/Cas9 system by disrupting MSTN and FGF5, resulting in expected phenotypes; for instance, higher body weight in MSTN-disrupted animals and increased fiber length in FGF5-disurpted animals. We provided adequate evidence to illustrate that the gene modifications induced by the disruption of FGF5 did occur at both morphological and genetic levels. In addition, the knockout alleles were capable of germline transmission. Together, with studies performed in other gene-modified livestock species such as pigs, our results demonstrate that the CRISPR/ Cas9 system has the potential to become a robust and efficient gene engineering tool in farm animals, and therefore will be critically important and applicable to breeding purposes.

Paper 27: Clonally derived chicken primordial germ cell lines maintain biological characteristics and proliferative potential in long-term culture

Chicken primordial germ cells (PGCs) are important cells with significant implications in preserving genetic resources, chicken breeding and production, and basic research on genetics and development. Currently, chicken PGCs can be cultured long-term in vitro to produce single-cell clones. However, systematic exploration of the cellular characteristics of these single-cell clonal lines has yet to be conducted. In this study, single-cell clonal lines were established from male and female PGCs of Rugao Yellow Chicken and Shouguang Black Chicken, respectively, using a micropipette-based method for single-cell isolation and culture. Analysis of glycogen granule staining, mRNA expression of pluripotency marker genes (*POUV*, *SOX2*, *NANOG*), germ cell marker genes (*DAZL*, *CVH*), and SSEA-1, EMA-1, SOX2, *C*-KIT, and CVH protein expression showed positive results, indicating that PGCs maintain normal cellular properties after single-cell cloning. Furthermore, tests on proliferation ability and gene expression levels in PGC single-cell clonal lines showed high expression of the pluripotency-related genes and TERT compared to control PGCs, and PGC single-cell clonal lines demonstrated higher proliferation ability. Finally, green fluorescent protein (GFP)-PGC single-cell clonal lines were established, and it was found that these single-cell clonal lines could still migrate into the gonads of recipients, suggesting their potential for germ-line transmission. This study systematically validated the normal cellular characteristics of PGC single-cell clonal lines, indicating that they could be applied in genetic modification research on chickens.

Paper 28: Progress and biotechnological prospects in fish transgenesis

The history of transgenesis is marked by milestones such as the development of cellular transdifferentiation, recombinant DNA, genetic modification of target cells, and finally, the generation of simpler genetically modified organisms (e.g. bacteria and mice). The first transgenic fish was developed in 1984, and since then, continuing technological advancements to improve gene transfer have led to more rapid, accurate, and efficient generation of transgenic animals. Among the established methods are microinjection, electroporation, lipofection, viral vectors, and gene targeting. Here, we review the history of animal transgenesis, with an emphasis on fish, in conjunction with major developments in genetic engineering over the past few decades. Importantly, spermatogonial stem cell modification and transplantation are two common techniques capable of revolutionizing the generation of transgenic fish. Furthermore, we discuss recent progress and future biotechnological prospects of fish transgenesis, which has strong applications for the aquaculture industry. Indeed, some transgenic fish are already available in the current market, validating continued efforts to improve economically important species with biotechnological advancements.

Paper 29: Potential of Genome Editing to Improve Aquaculture Breeding and Production

Aquaculture is the fastest growing food production sector and is rapidly becoming the primary source of seafood for human diets. Selective breeding programs are enabling genetic improvement of production traits, such as disease resistance, but progress is limited by the heritability of the trait and generation interval of the species. New breeding technologies, such as genome editing using CRISPR/Cas9 have the potential to expedite sustainable genetic improvement in aquaculture.

Genome editing can rapidly introduce favorable changes to the genome, such as fixing alleles at existing trait loci, creating de novo alleles, or introducing alleles from other strains or species. The high fecundity and external fertilization of most aquaculture species can facilitate genome editing for research and application at a scale that is not possible in farmed terrestrial animals.

Paper 30: Genome editing on finfish: Current status and implications for sustainability

Novel genome editing techniques allow for efficient and targeted improvement of aquaculture stock and might be a solution to solve challenges related to disease and environmental impacts. This review has retrieved the latest research on genome editing on aquacultured finfish species, exploring the technological progress and the scope. Genome editing has most often been used on Nile tilapia (Oreochromis niloticus Linnaeus), followed by Atlantic salmon (Salmo salar Linnaeus). More than half of the studies have focused on developing solutions for aquaculture challenges, while the rest can be characterized as basic research on fish genetics/physiology or technology development. Main traits researched are reproduction and development, growth, pigmentation, disease resistance, use of trans- GFP and study of the omega- 3 metabolism, respectively. There is a certain correlation between the species identified and their commercial relevance, indicating the relevance of most studies for present challenges of aquaculture. Reviewing geographical origin of the research, China has been in the forefront (29 publications), followed by the United States (9) and Norway (7). The research seems not to be dependent on regulative conditions in the respective countries, but merely on the purpose and objectives for the use of genome editing technologies. Some technical barriers identified in the studies are presented together with solutions to overcome these- off- target effects, ancestral genome duplication and mosaicism in F0. One of the objectives for use is the contribution to a more sustainable aquaculture, where the most prominent issues are solutions that contribute to minimizing impact on biodiversity.

Paper 31: Genome editing and its applications in genetic improvement in aquaculture

In the aquaculture industry, selective breeding has played an important role in increasing aquaculture production significantly. To meet the global growing demand for high quality proteins, it is essential to apply novel technologies to accelerate breeding to facilitate the increase in aquaculture production. Gene and genome editing technologies, including ZFNs, TALLENS and Crispr/Cas9, are promising tools to speed up genetic improvement. Among all the genome editing approaches, the CRISPR/Cas9 system is faster, cheaper and precise in editing genes/genomes. Therefore, the application of CRISPR/Cas9 technology in the editing of genomes in aquaculture species is emerging rapidly. It has been applied to precisely edit genes to identify gene functions and generate the preferred traits in over 20 aquaculture species. This review summa rises the genome editing technologies and their applications in the rapid improvement of economic traits in the aquaculture industry. Challenges and future directions of genome editing are also discussed.

Paper 32: Harnessing genomics to fast- track genetic improvement in aquaculture

Aquaculture is the fastest- growing farmed food sector and will soon become the primary source of fish and shellfish for human diets. In contrast to crop and livestock production, aquaculture production is derived from numerous, exceptionally diverse species that are typically in the early

stages of domestication. Genetic improvement of production traits via well- designed, managed breeding programmes has great potential to help meet the rising seafood demand driven by human population growth. Supported by continuous advances in sequencing and bioinformatics, genomics is increasingly being applied across the broad range of aquaculture species and at all stages of the domestication process to optimize selective breeding. In the future, combining genomic selection with biotechnological innovations, such as genome editing and surrogate broodstock technologies, may further expedite genetic improvement in aquaculture.

Paper 33: CRISPR/Cas Genome Editing—Can It Become a Game Changer in Future Fisheries Sector?

Fisheries and aquaculture are the fastest-growing food-producing sector and rapidly becoming an important element for the global food security since they are the primary source of seafood and high animal protein in the human diet. Genome editing offers new possibilities such as the clustered regularly interspaced palindromic repeats (CRISPR)/ CRISPR-associated protein (Cas9) technology, which has the potential to accelerate the sustainable genetic improvement in fisheries and aquaculture. The CRISPR/Cas9 system has four key components, namely, target DNA, Cas9, the protospacer adjacent motif sequence, and the guide RNA or single-guide RNA. CRISPR/Cas is cheaper, easier, and more precise than the other genome editing technologies and can be used as a new breeding technology in fisheries and aquaculture to solve the far-reaching challenges. The attributes like high fecundity, external fertilization, short generation interval, the established method of breeding, and the larval rearing of most aquaculture species have advantages for CRISPR/Cas9 genome editing applications. CRISPR/Cas9 has recently been applied to the traits valued in some aquaculture species (almost >20 species), targeting the main traits of traditional genetic improvement initiatives like growth, disease resistance, reproduction, sterility, and pigmentation. Genome editing can fast forward the breeding process with precision where changes occur in the targeted genes. The probability of desired changes occurring and passing the trait in the next generation is high, so it takes 1-3 generations to establish a breed. Moreover, CRISPR/Cas genome editing rapidly introduces favorable changes by disrupting genes with targeted minor changes, in contrast to transgenesis, which introduces foreign genes into the host genome and thereby alleviates major public concerns on safety. Although the CRISPR/Cas technology has a tremendous potential, there are several technical challenges and regulatory and public issues concerning the applications in fisheries and the aquaculture breeding sector. Nonetheless, the exciting point in the CRISPR/Cas9 genome editing is that two CRISPR edited fish, namely, red sea bream and tiger puffer developed by the Kyoto-based startup got approval and are now on the market for sale, and another fish, FLT-01 Nile tilapia developed by the AquaBounty, is not classified under genetically modified organism regulatory. However, there is still a way to go before it revolutionizes and becomes viable in commercial aquaculture as the new breeding technology for aquaculture-important traits and species.

Paper 34: Enhancing aquaculture disease resistance: Antimicrobial peptides and gene editing

Previous studies have demonstrated that CRISPR/Cas9-mediated genome editing and transgenesis by integrating vector-engineered antimicrobial peptide genes (AMGs) are effective to modulate the fish's innate immune system. To generalise the knowledge of AMG application in aquaculture, we recruited 544 data entries from a pool of empirical studies, which included 23 studies (two unpublished from our team) spanning 12 diseases. We systematically reprocessed and re-analysed these data by harnessing a cross-disease meta-analysis. On aggregate, AMG-genetic engineering aimed at enhancing disease resistance was shown to decrease the number of colony-forming units of bacteria from fish tissues, increase post-infection survival rates, and alter the expression of AMGs and immune-related genes. Further, AMG pathogen-combating activity was triggered within 2 h after infection and lasted 48 h. The over-expression of AMGs was highest in the spleen and skin, followed by the kidney and liver during this period. Typically, regardless of the type of AMGs, the synergistic expression of AMGs with IL-10, IKβ, TGFβ, C3b and TLR genes in AMG-integrated fish contributed to activating inflammatory/immune responses against pathogens. Our findings revealed that the efficiency of transgenic AMGs against pathogens was fish-, pathogen- and AMG-specific. Compared to bacteria, transgenic AMGs were less inhibitory to viruses and parasites. In addition, innovative CRISPR/Cas9-based transgenesis enabling the site-directed knock-in of foreign genes at multiple loci was assessed for the enhancement of disease resistance in combination with other favourable fish-production traits, including fast growth, sterility and enriched fatty-acid content. Altogether, our findings indicated that AMGs as transgenes have substantial promise to modulate the fish's innate immune system and enhance disease resistance.

Paper 35: A future for transgenic livestock

The techniques that are used to generate transgenic livestock are inefficient and expensive. This, coupled with the fact that most agriculturally relevant traits are complex and controlled by more than one gene, has restricted the use of transgenic technology. New methods for modifying the genome will underpin a resurgence of research using transgenic livestock. This will not only increase our understanding of basic biology in commercial species, but might also lead to the generation of animals that are more resistant to infectious disease.

Paper 36: Advances in farm animal transgenesis

The first transgenic livestock were produced in 1985 by microinjection of foreign DNA into zygotic pronuclei. This was the method of choice for more than 20 years, but more efficient protocols are now available, including somatic cell nuclear transfer and lentiviral transgenesis. Typical applications include carcass composition, lactational performance and wool production, as well as enhanced disease resistance and reduced environmental impact. Transgenic farm animal production for biomedical applications has found broad acceptance. In 2006 the European Medicines Agency (EMA) approved commercialization of the first recombinant pharmaceutical protein, antithrombin, produced in the mammary gland of transgenic goats. As the genome sequencing projects for various farm animal species are completed, it has become feasible to perform precise genetic modifications employing the emerging tools of lentiviral vectors, small interfering ribonucleic acids, mega nucleases, zinc finger nucleases and transposons. We anticipate

that genetic modification of farm animals will be instrumental in meeting global challenges in agricultural production and will open new horizons in biomedicine.

Paper 37: Genome editing and genetic engineering in livestock for advancing agricultural and biomedical applications

Genetic modification of livestock has a long standing and successful history, starting with domestication several thousand years ago. Modern animal breeding strategies predominantly based on marker-assisted and genomic selection, artificial insemination, and embryo transfer have led to significant improvement in the performance of domestic animals, and are the basis for regular supply of high quality animal derived food. However, the current strategy of breeding animals over multiple generations to introduce novel traits is not realistic in responding to the unprecedented challenges such as changing climate, pandemic diseases, and feeding an anticipated 3 billion increase in global population in the next three decades. Consequently, sophisticated genetic modifications that allow for seamless introgression of novel alleles or traits and introduction of precise modifications without affecting the overall genetic merit of the animal are required for addressing these pressing challenges. The requirement for precise modifications is especially important in the context of modeling human diseases for the development of therapeutic interventions. The animal science community envisions the genome editors as essential tools in addressing these critical priorities in agriculture and biomedicine, and for advancing livestock genetic engineering for agriculture, biomedical as well as “dual purpose” applications.

Paper 38: Livestock 2.0– genome editing for fitter, healthier, and more productive farmed animals

The human population is growing, and as a result we need to produce more food whilst reducing the impact of farming on the environment. Selective breeding and genomic selection have had a transformational impact on livestock productivity, and now transgenic and genome-editing technologies offer exciting opportunities for the production of fitter, healthier and more-productive livestock. Here, we review recent progress in the application of genome editing to farmed animal species and discuss the potential impact on our ability to produce food.

Paper 39: Genome editing approaches to augment livestock breeding programs

The prospect of genome editing offers a number of promising opportunities for livestock breeders. Firstly, these tools can be used in functional genomics to elucidate gene function, and identify causal variants underlying monogenic traits. Secondly, they can be used to precisely introduce useful genetic variation into structured livestock breeding programs. Such variation may include repair of genetic defects, the inactivation of undesired genes, and the moving of useful alleles and haplotypes between breeds in the absence of linkage drag. Editing could also be used to accelerate the rate of genetic progress by enabling the replacement of the germ cell lineage of commercial breeding animals with cells derived from genetically elite lines. In the future, editing may also provide a useful complement to evolving approaches to decrease the length of the generation interval through in vitro generation of gametes. For editing to be adopted, it will need to seamlessly integrate with livestock breeding schemes. This will likely involve introducing edits into multiple

elite animals to avoid genetic bottlenecks. It will also require editing of different breeds and lines to maintain genetic diversity, and enable structured cross breeding. This requirement is at odds with the process-based trigger and event-based regulatory approach that has been proposed for the products of genome editing by several countries. In the absence of regulatory harmony, researchers in some countries will have the ability to use genome editing in food animals, while others will not, resulting in disparate access to these tools, and ultimately the potential for global trade disruptions.

Paper 40: Precise Genome Editing in Poultry and Its Application to Industries

Poultry such as chickens are valuable model animals not only in the food industry, but also in developmental biology and biomedicine. Recently, precise genome-editing technologies mediated by the CRISPR/Cas9 system have developed rapidly, enabling the production of genome-edited poultry models with novel traits that are applicable to basic sciences, agriculture, and biomedical industry. In particular, these techniques have been combined with cultured primordial germ cells (PGCs) and viral vector systems to generate a valuable genome-edited avian model for a variety of purposes. Here, we summarize recent progress in CRISPR/Cas9-based genome-editing technology and its applications to avian species. In addition, we describe further applications of genome-edited poultry in various industries.

Paper 41: Recent Advances in the Application of CRISPR/Cas9 Gene Editing System in Poultry Species

CRISPR/Cas9 system genome editing is revolutionizing genetics research in a wide spectrum of animal models in the genetic era. Among these animals, is the poultry species. CRISPR technology is the newest and most advanced gene-editing tool that allows researchers to modify and alter gene functions for transcriptional regulation, gene targeting, epigenetic modification, gene therapy, and drug delivery in the animal genome. The applicability of the CRISPR/Cas9 system in gene editing and modification of genomes in the avian species is still emerging. Up to date, substantial progress in using CRISPR/ Cas9 technology has been made in only two poultry species (chicken and quail), with chicken taking the lead. There have been major recent advances in the modification of the avian genome through their germ cell lineages. In the poultry industry, breeders and producers can utilize CRISPR-mediated approaches to enhance the many required genetic variations towards the poultry population that are absent in a given poultry flock. Thus, CRISPR allows the benefit of accessing genetic characteristics that cannot otherwise be used for poultry production. Therefore CRISPR/Cas9 becomes a very powerful and robust tool for editing genes that allow for the introduction or regulation of genetic information in poultry genomes. However, the CRISPR/Cas9 technology has several limitations that need to be addressed to enhance its use in the poultry industry. This review evaluates and provides a summary of recent advances in applying CRISPR/Cas9 gene editing technology in poultry research and explores its potential use in advancing poultry breeding and production with a major focus on chicken and quail. This could aid future advancements in the use of CRISPR technology to improve poultry production.

Paper 42: Advances and Applications of Transgenesis in Farm Animals

Modification of animal genomes is an age-old tradition rooted in selective breeding of genetic outlier animals over successive generations for enhancing production traits. However, selective breeding has limitations; chief among them is the requirement for the existence of a beneficial trait within the population, low heritability of traits, and the long time required for mating over multiple generations to “fix” the trait within a population. Given the need to sustainably increase animal production to feed the anticipated 9 billion global population by 2030, and a looming threat from climate change, there is a pressing need for animal agriculture to be more precise and responsive than selective breeding. This includes utilizing all the tools at our disposal such as transgenesis and genome editing. The emergence of genome engineering tools like meganucleases, zincfinger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and clustered regularly interspaced short palindromic repeats/Cas (CRISPR/Cas) system allows us to introduce precise genetic modification at nucleotide resolution while also facilitating large transgene integration. Concordant with the developments in genomic sequencing, progress among transgenic approaches has reached feverish pace. The current manuscript reviews past and current developments in gene transfer techniques, including microinjection method, sperm mediated gene transfer, cytoplasmic DNA injection, cloning/ somatic cell nuclear transfer (SCNT), embryonic stem cell, and retroviral vector mediated methods to improve efficiency of genetic engineering, while also focusing on novel genome editing tools like designer nucleases for the generation of transgenic animals. Cumulatively, these tools provide a better platform to produce transgenic animals for addressing critical priorities of animal agriculture and biomedical research.

Paper 43: Molecular breeding of farm animals through gene editing

The rapid development of biotechnology has facilitated our understanding of the biological functions of candidate genes for important economic traits in farm animals. Molecular breeding by gene editing has greatly revolutionized the breeding of farm animals. Through gene editing and embryo manipulation, breeds with designed economic or disease-resistant traits can be readily generated. Along with this fast progress, the safety assessment of gene-edited farm animals has attracted public and regulatory attention. This review summarizes the research progress of gene editing in farm animals, focusing on performance improvement, disease resistance, bioreactors, animal welfare, and environmental friendliness. The limitations and future development of gene editing technology in farm animal breeding are also discussed.

Paper 44: Production of cloned transgenic cow expressing omega-3 fatty acids

n-3 Polyunsaturated fatty acids (n-3PUFA) are important for human health. Alternative resources of n-3 PUAFs created by transgenic domestic animals would be an economic approach. In this study, we generated a mfat-1 transgenic cattle expressed a Caenorhabditis elegans gene, mfat-1, encoding an n-3 fatty acid desaturase. Fatty acids analysis of tissue and milk showed that all of the examined n-3 PUAFs were greatly increased and simultaneously the n-6 PUAFs decreased in the transgenic cow. A significantly reduction of n-6/n-3 ratios ($P<0.05$) in both tissue and milk were observed.

Paper 45: Interaction of diet and the masou salmon D5-desaturase transgene on D6-desaturase and stearoyl-CoA desaturase gene expression and N-3 fatty acid level in common carp (Cyprinus carpio)

The masou salmon D5-desaturase-like gene (D5D) driven by the common carp b-actin promoter was transferred into common carp (Cyprinus carpio) that were fed two diets. For P1 transgenic fish fed a commercial diet, D6-desaturase-like gene (D6D) and stearoyl-CoA desaturase (SCD) mRNA levels in muscle were up-regulated 12.7- and 17.9 fold, respectively, and the D6D mRNA level in the gonad of transgenic fish was up-regulated 6.9-fold compared to that of non-transgenic fish. In contrast, D6D and SCD mRNA levels in transgenic fish were dramatically down-regulated, 50.2- and 16.7-fold in brain, and 5.4- and 2.4-fold in liver, respectively, in comparison with those of non transgenic fish. When fed a specially formulated diet, D6D and SCD mRNA levels in muscle of transgenic fish were up-regulated 41.5- and 8.9-fold, respectively, and in liver 6.0- and 3.3-fold, respectively, compared to those of non-transgenic fish. In contrast, D6D and SCD mRNA levels in the gonad of transgenic fish were down-regulated 5.5 and 12.4-fold, respectively, and D6D and SCD mRNA levels in the brain were down-regulated 14.9- and 1.4 fold, respectively, compared to those of non-transgenic fish. The transgenic common carp fed the commercial diet had 1.07-fold EPA, 1.12-fold DPA, 1.07-fold DHA, and 1.07-fold higher observed total omega-3 fatty acid levels than non-transgenic common carp. Although these differences were not statistically different, there were significantly higher omega-3 fatty acid levels when considering the differences for all of the individual omega-3 fatty acids. The genotype and diet interactions observed indicated that the potential of desaturase transgenesis cannot be realized without using a well-designed diet with the needed amount of substrates.

Paper 46: Precise gene editing paves the way for derivation of Mannheimia haemolytica leukotoxin-resistant cattle

Signal peptides of membrane proteins are cleaved by signal peptidase once the nascent proteins reach the endoplasmic reticulum. Previously, we reported that, contrary to the paradigm, the signal peptide of ruminant CD18, the β subunit of β2 integrins, is not cleaved and hence remains intact on mature CD18 molecules expressed on the surface of ruminant leukocytes. Leukotoxin secreted by Mannheimia (Pasteur ella) haemolytica binds to the intact signal peptide and causes cytolysis of ruminant leukocytes, resulting in acute inflammation and lung tissue damage. We also demonstrated that site-directed mutagenesis leading to substitution of cleavage-inhibiting glutamine (Q), at amino acid position 5 upstream of the signal peptide cleavage site, with cleavage-inducing glycine (G) results in the cleavage of the signal peptide and abrogation of leukotoxin-induced cytolysis of target cells. In this proof-of-principle study, we used precise gene editing to induce Q(−5)G substitution in both alleles of CD18inbovinefetal fibroblast cells. The gene-edited fibroblasts were used for somatic nuclear transfer and cloning to produce a bovine fetus homozygous for the Q(−5)G substitution. The leukocyte population of this engineered ruminant expressed CD18 without the signal peptide. More importantly, these leukocytes were absolutely resistant to leukotoxin-induced cytolysis. This report demonstrates the feasibility of developing

lines of cattle genetically resistant to M. haemolytica-caused pneumonia, which inflicts an economic loss of over $1 billion to the US cattle industry alone.

Paper 47: Integration of alligator cathelicidin gene via two CRISPR/Cas9-assisted systems enhances bacterial resistance in blue catfish, Ictalurus furcatus

CRISPR/Cas9-mediated genome editing has paved new avenues for improving production-valued traits in aquaculture by knocking out or disrupting functional genes. However, utilizing CRISPR/Cas9-based knock-in (KI) of exogenous genes can expedite genetic improvement of traits such as disease resistance, which remains problematic in farmed fish. In this study, we successfully generated transgenic blue catfish (Ictalurus furcatus) of primarily Rio Grande strain ancestry with site-specific KI of the alligator (Alligator sinensis) cathelicidin (As-Cath) gene into the luteinizing hormone (lh) locus via two CRISPR/Cas9-mediated KI systems, assisted by the linear double-stranded DNA (dsDNA) and double-cut plasmid, respectively. High integration rates were observed with linear dsDNA (16.67%, [13/78]) and double-cut plasmid strategies (24.53%, [26/106]). In addition, the on- target KI efficiency of the double-cut plasmid strategy (16.04%, [17/106]) was 1.67 times higher than that of the linear dsDNA strategy (10.26%, [8/78]) based on the odds ratio. The relative expression of the As-Cath transgene of P 1 founders was detected in nine tissues, dominated by the kidney, skin, and muscle (14.30-, 7.71- and 6.92-fold change, $P < 0.05$). Moreover, the As-Cath transgenic blue catfish showed a higher cumulative survival rate than that of wild-type controls (80% vs. 30%, $P < 0.05$) following Flavobacterium covae infection. Survival during culture supports the challenge data as survival of As-Cath transgenic individuals was 97.1% while that of pooled non-transgenic individuals was observed to be less 87.0% ($P = 0.15$). The growth rates and external morphology of the transgenic and wild-type siblings were not different ($P > 0.05$), indicating no pleiotropic effects of the As-Cath transgene integration at the lh locus in the P 1 founders for this trait. Taken together, our findings demonstrate that CRISPR/Cas9-assisted KI of an antimicrobial peptide gene can be ach ieved in blue catfish with high integration efficiency, and As-Cath transgenic blue catfish have improved disease resistance, which is a promising strategy for disease reduction in aquaculture.

Paper 48: Generation of myostatin-knockout chickens mediated by D10A-Cas9 nickase

Many studies have been conducted to improve economically important livestock traits such as feed efficiency and muscle growth. Genome editing technologies represent a major advancement for both basic research and agronomic biotechnology development. The clustered regularly interspaced short palindromic repeats (CRISPR)/ Cas9 technical platform is a powerful tool used to engineer specific targeted loci. However, the potential occurrence of off-target effects, including the cleavage of unintended targets, limits the practical applications of Cas9-mediated genome editing. In this study, to minimize the off-target effects of this technology, we utilized D10A Cas9 nickase to generate myostatin-knockout (MSTN KO) chickens via primordial germ cells. D10A-Cas9 nickase (Cas9n)-mediated MSTN KO chickens exhibited significantly larger skeletal muscles in the breast and leg. Degrees of skeletal muscle hypertrophy and hyperplasia induced by myostatin deletion differed by sex and muscle type. The abdominal fat deposition was dramatically

lower in MSTN KO chickens than in wild-type chickens. Our results demonstrate that the D10A-Cas9 technical platform can facilitate precise and efficient targeted genome engineering and may broaden the range of applications for genome-edited chickens in practical industrialization and as animal models of human diseases.

Paper 49: Generation of fast growth Nile tilapia (Oreochromis niloticus) by myostatin gene mutation

Tilapia is the second most prolific species grown in aquaculture after carp, and is widely grown in >100 countries. Myostatin (MSTN) has been proved to be a negative regulator of skeletal muscle growth. Mutation of MSTN gene resulted in significant increase in both body size and muscle mass in vertebrates, mostly with a species-specific effect. To generate a new strain in Nile tilapia (Oreochromis niloticus) with fast growth traits, the molecular characterization, gene expression and functional studies of mstn gene were extensively investigated. Phylogenetic and synteny analyses indicated that mstnb, but not mstna, might be the orthologous gene of mammalian MSTN gene. Expression pattern analyzed by qRT-PCR demonstrated that mstnb is abundant in muscle, while mstna is dominantly expressed in the brain. Fluorescence in situ hybridization (FISH) analysis also demonstrated that mstnb is exclusively expressed in the basement membrane of white skeletal muscle fibers, further suggesting the possible role of mstnb gene in tilapia muscle growth. To verify the roles of mstnb in skeletal muscle growth, we obtain the mstnb/wild type (WT) fish, the mstnb / mutant using CRISPR/Cas9 gene editing technology. Compared with the mutants showed a typical double-muscle phenotype with increased muscle mass, sticking out between head and dorsal fin, from 5 months after hatching (mah). Morphological observation revealed an excessive proliferation of white muscle fibers at 7 mah. The average body weights (21.67 ± 8.26 g), body heights (4.46 ± 0.65 cm) and body widths (2.36 ± 0.53 cm) of the mstnb / 37.21% higher than those of the mstnb +/+ f ish were 49.45%, 32.74% and f ish (body weight, 14.50 ± 8.60 g; body height, 3.36 ± 0.45 cm; body width, 1.72 ± 0.18 cm) at 5 mah. The results showed that the growth performance parameters of the mstnb / f ish, including weight gain rate (1.99 times), condition factor (1.77 times) and specific growth rate (1.23 times), were significantly higher than the mstnb +/+ fish under laboratory conditions at 5 mah. In addition, the parameters of WGR, CF, SGR and FE were significantly increased in mstnb / / Nile tilapia than those of mstnb Nile tilapia and GIFT tilapia for a 90-day feeding trial under the wild natural environment. Interestingly, sexually dimorphic effects on muscle growth have been found with an increase of growth performance of skeletal muscle in mstn XY fish, but not mstn / +/+ XX fish at 7 mah. Taken together, this study, for the first time, proved that mstn gene mutation might promote the generation of new fast-growing male strain of Nile tilapia.

Paper 50: Growth and Tissue Accretion Rates of Swine Expressing an Insulin-like Growth Factor I Transgene

The goal of this research was to determine whether directing expression of an insulin-like growth factor I (IGF-I) transgene specifically to striated muscle would alter the growth characteristics in swine. Transgenic pigs were produced with a fusion gene composed of avian skeletal a-actin

regulatory sequences and a cDNA encoding human IGF-I. Six founder transgenic pigs were mated to nontransgenic pigs to produce 11 litters of G1 transgenic and sibling control progeny. Birth weight, weaning weight, and proportion of pig survival did not differ between transgenic and control pigs. The ADG of pigs as they grew incrementally from 20 to 60kg, 60 to 90kg, and 90 to 120kg, respectively, did not significantly differ between transgenic and control pigs. Efficiency of feed utilization (gain: feed) was also similar for transgenic and control pigs. Plasma IGF-I and porcine growth hormone (pGH) concentrations were determined at 60, 90, and 120kg body weight. Plasma IGF-I concentrations were 19% higher in transgenic gilts than control gilts and 11.1% higher in transgenic boars than control boars (P¼0.0005). Plasma IGF-I concentrations for boars were also higher than for gilts (P¼0.0001). At 60, 90, and 120kg body weight each pig was scanned by dual energy X-ray absorptiometry (DXA) to derive comparative estimates of carcass fat, lean, bone content of the live animal. Control pigs had more fat and less lean tissue than transgenic pigs at each of the scanning periods and the difference became more pronounced as the pigs grew heavier. Transgenic pigs also had a slightly lower percentage of bone than control pigs. While daily rates of lean tissue accretion did not differ for transgenic and control pigs, daily rates of fat accretion were lower in transgenic pigs than in control pigs. Based on these results we conclude that expression of IGF-I in the skeletal muscles gradually altered body composition as pigs became older but did not have a major affect on growth performance.