Promotion of Alleles by Genome Engineering

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Abstract: Traditional genetic selection programs based on pedigree and performance information have been used to improve livestock populations for decades. The introduction of high-density single nucleotide polymorphism genotyping about 10 years ago supported increased rates of gain through more accurate prediction of genetic merit earlier in life. Recent continued technological advances enable the routine use of genetic engineering and gene editing tools in livestock research and, increasingly, production systems. Livestock geneticists have responded by proposing new breeding schemes that combine traditional selection methodology with these new tools to substantially increase rates of genetic gain while reducing harmful effects due to decreased heterozygosity. Genetic improvement strategies based on gene drives have the potential further increase rates of gain but pose risks that may not be acceptable to the public. Intense debate about the use of these technologies in the animal food chain are being driven by regulatory agencies and consumer advocates, and it is not clear if genetically modified animals will be acceptable to consumers. This review focuses on the application of genetic engineering and genome engineering tools to livestock population improvement through the management of genetic load and the promotion of desirable alleles in the population associated with both monogenic and polygenic traits. Limitations of the current technology, such as limited knowledge of true causal variants, are discussed, as are regulatory and consumer acceptance issues.

Keywords: gene editing, genome engineering, quantitative traits, recessive disorders

Review Methodology: Existing recent reviews on the subject have served as a useful input. More recent literature has been searched using Google Scholar. Search terms used: gene edited cattle; gene editing in livestock; genetic engineering in livestock; transgenic cattle; transgenic livestock. Published and unpublished information from researchers in the field has been accessed through personal communication.

Purpose of this Review

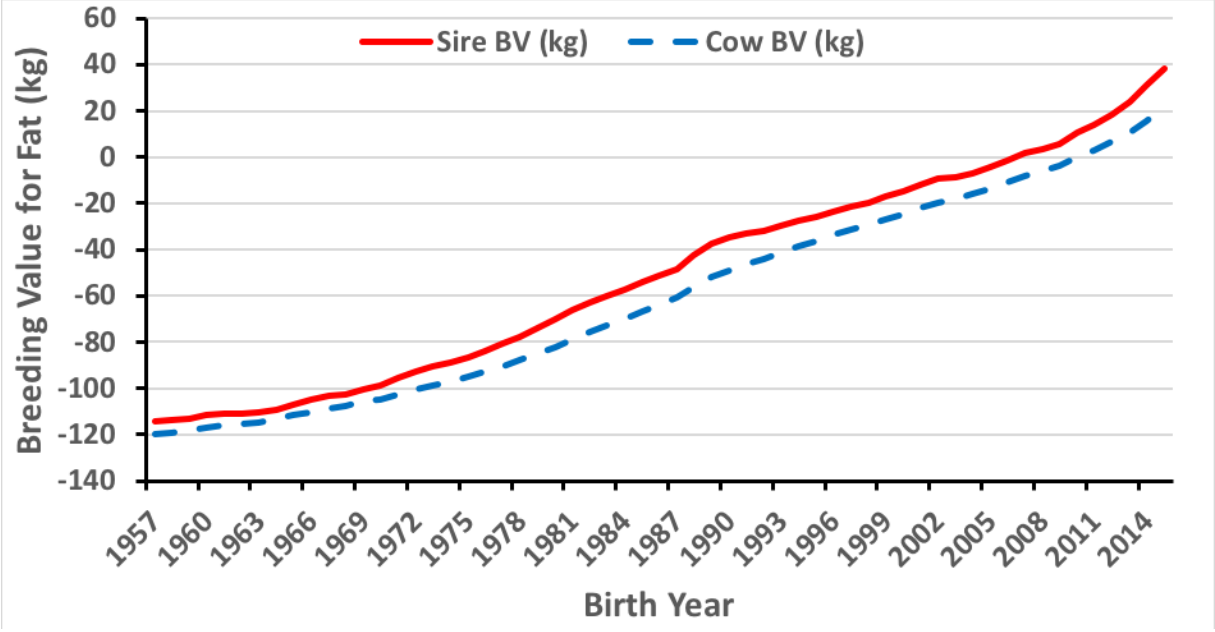
The objective of this review is to discuss the use of modern genome engineering tools to improve the genetic merit of livestock populations for monogenic and polygenic traits.

Genetic Improvement of Livestock

Both traditional genetic selection, which is based on the performance of relatives, and genomic selection, which uses single nucleotide polymorphism genotypes to directly track DNA inherited from parents, have been used very successfully to improve the genetic merit of many traits in a number of different populations. The most successful application of these technologies may be in dairy cattle [1], but they also have been used to improve many other livestock (e.g., [2,3]) and plant [4] species.

Traditional genetic selection programs have focused on improving the average genetic merit of animals in the population each generation. For example, the number of cows in the US national dairy herd has decreased from 23.67 million in 1940 to 9.26 million in 2014, while average annual fat yield has increased from 216 kg to 489 kg over the same time period. Much of this improvement in productivity is due to selection, with genetic gain averaging almost 2.4 kg of fat per year between 1960 and 2015 (Figure 1), roughly half of the total improvement in fat yield observed over that time period.

Figure 1. Genetic trend for predicted transmitting ability (PTA) for fat yield of US Holstein bulls (solid red line) and cows (dashed blue line) between 1957 and 2015.



Source: Trend in Fat BV for Holstein or Red & White Calculated April 2018 (https://queries.uscdcb.com/eval/summary/trend.cfm?R_Menu=HO.f#StartBody).

This is done by selecting parents that have high genetic merit for traits of interest to produce the next generation of animals. The breeder's equation, shown below, describes how different aspects of traits under selection affect the rate of genetic gain in a population (e.g., [5]).

$$\Delta G_{year} = \frac{\sqrt{reliability} \times selection\ intensity \times \sqrt{genetic\ variance}}{generation\ interval}$$

In this equation, "ΔG_{year}" is the annual rate of genetic change in the population, "reliability" is a measure of the precision with which an individual's genetic merit is estimated, "selection intensity" is a measure of how selectively the parents of the next generation are chosen, "genetic variance" is the proportion of variation among animals in the population that is attributable to genetic differences, and "generation interval" is the average age of parents when their offspring are born. The "reliability" and "selection intensity" terms are the easiest to manipulate in traditional breeding programs, subject to market constraints. Genetic engineering and genomic selection provide opportunities to increase rates of gain through the "genetic variance" and "generation interval" terms, as well. These distinctions are not absolute; for example, generation interval can be reduced in a traditional breeding program if breeders are willing to accept lower reliabilities.

Genetic and Genome Engineering of Livestock

Introgression of an allele into a population by traditional breeding is typically a very slow process. The use of genetic and genome engineering technologies can dramatically increase the rate of introgression of desirable alleles [6]. In the discussion that follows, "genetic engineering" refers to the insertion of exogenous DNA into an animal's genome, while "genome engineering" refers to manipulation of an animal's own DNA.

Genetic Engineering Through Transgenesis

The first transgenic livestock were created by microinjecting DNA coding for a metallothionein I-human growth hormone fusion gene into embryos of pigs, rabbits, and sheep [7]. Since those first successes, a number of transgenic animals and fish have been developed (see [8] and [9] for recent reviews). The production of transgenic animals remains laborious and expensive even though the microinjection of zygotes has been replaced by somatic cell nuclear transfer [8]. Animals have been modified both for food purposes, such as increased health and longevity (e.g., [10]), and non-food purposes, such as the expression of desirable products in their milk (e.g., [11,12]).

Table 1. Some examples of cattle genetically engineered for agricultural and pharmaceutical production.

Gene	Source	Description	Reference
β- and κ-casein	Cattle	Enhancement of milk composition and processing efficiency	[13]
Lactoferrin	Human	Innate host (immune) defense	[14]
Lysostaphin	*S. simulans*	Resistance to *S. aureus* mastitis	[10]
Myostatin	Knockout	Increased muscle growth	[15]

Omega-3 fatty acids	*C. elegans*	Improved human health through desirable fatty acid composition of milk	[16]

There are both technical [17] and ethical [18,19] challenges associated with the widespread use of transgenic animals in livestock production, although the use of animal bioreactors to produce biomedical materials remains appealing (e.g., [20,21]). In addition, it remains expensive to generate transgenic animals because efficiency is low and control over transgene integration remains poor. Given such technical and economic limitations, transgenic livestock are likely to be used only in limited, biomedical settings.

Genome Engineering Through Precision Gene Editing

A number of tools are now available for editing eukaryotic genomes, including clustered regularly interspaced short palindromic repeats (CRISPR; [22]) , transcription activator-like effector nucleases (TALEN; [23]), and zinc finger nucleases (ZFN; [24]). Edits can include the deactivation (knock-out) of genes, the alteration of short segments of DNA, and the insertion of new genes. The latter application means that the line between traditional genetic engineering and genome engineering is somewhat blurry since both tools can be used to introduce DNA from one species into another. However, gene editing refers to a specific suite of tools for making targeted changes to DNA, while genome engineering broadly refers to a wide array of strategies for using gene editing in concert with breeding strategies or other technologies to make population-wide changes [25].

Current gene editing approaches are based on the use of site-directed nucleases to precisely introduce double stranded breaks (DSB) at predetermined locations in the genome (e.g., [26]). There are two distinct pathways for repairing DSB. The underlying principle is that host repair factors will congregate at the site of a DSB, where they will repair the DNA. In the absence of a guide sequence to provide a template, the broken double-stranded sequence will be repaired by the non-homologous end-joining pathway (NHEJ), a process known as targeted mutagenesis, which often introduces deletions or additions of a few base pairs and can result in gene knock-outs [27]. However, if a nucleic acid template (donor DNA) is provided, then the repair enzymes will use the donor DNA as a guide for precise repair by homologous recombination (HR). The HR pathway can be used to precisely add, delete, or replace letters in the genetic code at the location of the break [27]. The ZFN-, TALEN-, and CRISPR-based approaches are briefly described below, but interested readers should consult a more comprehensive source such as [26] for additional details. Van Eenennaam [28] provides a comprehensive description of gene editing combined with somatic cell nuclear transfer in livestock production systems (see Figure 2 of that paper).

Zinc finger nucleases. Artificial restriction enzymes can be constructed by joining zinc finger domains, which bind to 3 to 6 nucleotide triplets, to nucleases that cut DNA [26]. The limited length of these domains can result in off-target breaks. The *Fok1* enzyme that is commonly used as the nuclease domain must be present as a dimer in order to induce double-stranded breaks, which means that multiple ZFN must be used to target non-palindromic sites.

Alleles can be knocked-out by using individual ZFN to target single base pairs, while multiple ZFN can be used to cut out a large piece of DNA. If a homologous (guide) template is provided then DNA can be inserted at the break site using homology-directed repair. There are many commercial providers of ZFN-based tools, making their use relatively simple and affordable. The greatest obstacle to the use of ZFN is the construction of sufficiently specific target domains. This tool has been used to produce gene-edited cattle [29] and pigs [30].

Transcription activator-like effector nucleases. More precise targeting of single base pairs can be achieved using transcription activator-like effector molecules ligated to nucleases to produce TALEN. Their principal advantage over ZFN is that the DNA-binding domains in TALE are composed of a series of 33- to 35-amino-acid repeats, resulting in greater specificity. The specific base targeted by the TALE is flanked by target sequence so that non-palindromic DNA may be targeted using a single TALEN, rather than multiple ZFN (e.g., [31]). This technology has been used to produce many gene-edited animals, including cattle [32], chickens [33], and pigs [30].

Clustered regularly interspaced short palindromic repeats. Clustered regularly interspaced short palindromic repeats. The construction of novel ZFN and TALEN can be time-consuming, labor-intensive, and costly. which depend on protein-DNA interactions, the CRISPR-Cas9 system is derived from RNA-based defense systems found in bacteria [34]. The Cas9 protein uses a sequence of guide RNA (gRNA) to identify DNA sequences of interest, such as genes. The gRNA binds to the complementary DNA sequence and a double-stranded break is made. Genes can then be knocked-out by NHEJ, or "faulty" genes can be repaired or new genes inserted using HR. However, an important limitation of CRISPR-Cas9 is that editing targets must be upstream of a protospacer adjacent motif (PAM), a 3 to 5 nucleotide motif that serves as a binding signal for the Cas9 protein. Alternative PAM have been identified in different species of bacteria (e.g., [35–37]), and Cas9 has been engineered to recognize a wider array of motifs [38,39]. CRISPR-Cas systems have several advantages over ZFN- and TALEN-based systems, including simplicity of designing target sequences (it is easy to design gRNA targets), efficiency (plasmids encoding Cas proteins and gRNA can be microinjected directly into embryos) [40] [40], and the ability to easily multiplex edits [41]. This approach has been used to produce gene-edited cattle [42], goats [43], and pigs [44].

There is great interest in the use of gene editing to improve animal health (e.g., [45]) and several studies have produced genetically modified cattle that are able to resist common diseases (Table 1). Additionally, gene editing may be an effective tool for reducing the frequency of genetic disorders in livestock populations or eliminating those disorders altogether (e.g., [46–48]), and a recent series of simulation studies showed that gene editing also has the potential to improve rates of genetic gain for quantitative traits [49,50].

Table 2. Examples of gene-edited cattle (adapted from [28]).

Gene	Type	Description	Reference
Beta-lactoglobulin	Knockout	Elimination of milk allergen	[51,52]

Lysostaphin	Transgene	Resistance to *S. aureus* mastitis	[53]
Lysozyme	Transgene	Resistance to *S. aureus* mastitis	[54]
Myostatin	Knockout	Increased muscle yield	[55,56]
NRAMP1	Insertion	Resistance to tuberculosis	[54]
POLLED	Substitution	Animals born without horns	[32,57]
PRNP	Knockout	Prion resistance	[58,59]
SP110	Transgene	Resistance to tuberculosis	[60]

Applications of Genetic and Genome Engineering in Livestock Breeding Programs

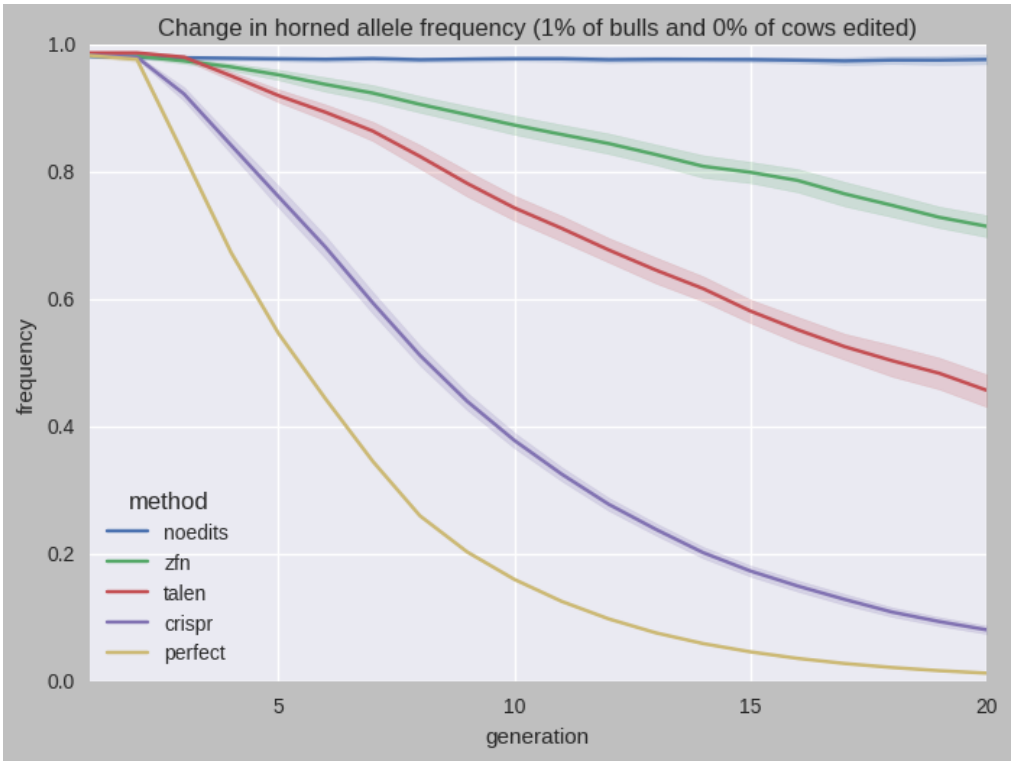
Traits of interest in livestock breeding programs are commonly grouped into two groups: those controlled by a single gene (monogenic traits) and those controlled by many genes acting together (polygenic traits). There are also cases, such as susceptibility to some diseases, in which a phenotype is influenced by many loci but a major gene also accounts for a large proportion of the genetic variance. Genetic and genome engineering are applied differently to mono- versus polygenic traits, as described below. Some applications proposed in the following discussion are not yet possible given the current state of the technology, and may never be feasible. Those cases will be noted, but the discussion will focus on possible benefits in the long term. Technical limitations and other concerns will be discussed in the section titled "Future outlook: opportunities and obstacles".

Monogenic Traits

Modern genetics began with the work of Mendel on simple traits in peas (*Pisum sativum*) [61], such as flower color and seed appearance. While many traits of economic importance in livestock are controlled by many genes acting together, many monogenic traits are important for animal health and welfare.

Polled. Not all recessive alleles of interest in livestock populations are lethal, with the polled locus (OMIA ID: 000483-9913) in cattle as a prominent example. The polled condition in cattle is due to the action of a dominant allele [62] with a very low frequency – less than 1 % – in the US Holstein population, and there is interest in increasing its frequency to improve animal welfare. However, polled Holstein bulls have lower average genetic merit than horned bulls and there have historically been relatively few polled bulls available. Gene editing can be used to produce polled calves from high-genetic-merit bulls; two healthy, homozygous polled Holstein calves, Spotigy and Buri, were produced by TALEN-stimulated homology-dependent repair [32]. Several studies have shown that that the frequency of the polled allele increases much faster using genome engineering than traditional breeding alone [46,48,63].

Figure 2. Observed allele frequency of the Holstein horned locus in a simulated population for traditional breeding ("noedits") and four different gene-editing technologies ("CRISPR", "TALEN", "ZFN", and a hypothetical "perfect" method) over 20 years when the top 1 % of bulls and no cows are edited (adapted from [46]). The methods differ in their rates of efficiency [30,64], which is why they produce different allele frequencies.



Source: https://github.com/wintermind/gene-editing/blob/master/gene-editing/Rate%20of%20horned%20frequency%20change%20(1%25%20bulls%2C%200%25%20cows%20edited).ipynb.

Slick. The slick hair gene (OMIA ID: 001372-9913), an autosomal dominant locus, confers increased thermotolerance through a short, thin hair coat. The slick gene has been introgressed from the Senepol breed, where it was first observed, into US Holsteins [65]. A series of convergent mutations in the prolactin and prolactin receptor genes [66,67] are responsible for the phenotype. The slick gene is an attractive target for genome engineering, because an intra-species allele transfer can be used to produce clones of high genetic merit bulls that also are slick. Daughters of such edited bulls would have lower rectal temperatures and respiration rates, higher sweating rates, and smaller decreases in milk yield under heat stress conditions than daughters of wild type bulls [65]. Application of gene editing to slick provides clear advantages to cows, consumers, and farmers.

Management of genetic load. Recessive disorders have been identified in livestock populations since animal breeding programs began, and more than 500 such disorders have been catalogued in cattle alone [68]. In the past, test matings were used to identify carriers of recessive disorders as part of progeny-test programs [69], but most recessive mutations were identified after a carrier bull sired many daughters and had sons used for artificial insemination (e.g., bovine leukocyte adhesion deficiency [70], complex vertebral malformation [71], and deficiency of uridine monophosphate synthase [72]). Newly arisen or previously unknown

recessives also can spread quickly through a population through the use of popular bulls before routine screening is possible because such defects are not directly observable (e.g., *CWC15* in the Jersey breed [73]).

Genomic tools have enabled the detection of new recessive alleles that have harmful effects on fertility [74–77], many of which act early in gestation and could not previously be distinguished from failed matings. MacArthur et al. [78] estimated that human genomes contain approximately 100 loss-of-function mutations, and about 20 completely inactivated genes. It seems reasonable to assume that other mammals with genomes of similar sizes may carry a similar genetic load. It is important to remember, however, that human populations are not subject to culling for fitness as livestock are. The apparent increase in the rate of discovery of genetic defects does not mean that mutations are increasing in frequency, it simply reflects our improved ability to detect such changes in the genome.

CRISPR-Cas9-based gene editing already has been used in human embryos to repair a mutation in *MYBPC3*, which causes hypertrophic cardiomyopathy [79], and similar approaches could be used to repair genetic defects in bulls with high genetic merit. Cole [46] has proposed that gene editing can be used to eliminate deleterious recessive alleles from dairy cattle populations, and showed that the rate of embryonic loss (death) decreases rapidly when the top 1 % of bulls in the population are edited. Johnson et al. [47] used simulation to determine how best to use gene editing to manage genetic load, concluding that selection against carriers may the best approach to managing recessive alleles in the short term, but that gene editing may be more effective in the long-term. However, Van Eenennaam and Kinghorn [80] found that selection against carriers may resulted in reduced rates of genetic gain. Another embryo-based strategy is to edit all IVF embryos and then genotype embryo biopsies to both confirm the success of the edit and identify the embryo with the highest genetic merit [81]. However, it may not make economic sense to edit individual embryos when IVF and embryo genotyping can be used to identify another embryo from the same flush with similar genetic merit that is free of known genetic defects. As the number of known genetic defects increases, it may not be possible to identify defect-free embryos from a flush, and gene editing may be necessary in order to produce animals free of known defects.

Validation of causal variants. Gene editing can be used to quickly and affordably validate causal variants associated with certain traits, such as early embryonic loss. For example, a loss-of-function mutation in the *CWC15* gene is thought to be causal for embryonic losses associated with the JH1 haplotype in the Jersey breed [73]. The biological mechanism has not been elucidated and the association has not been validated *in vitro*, although there are time-dependent differences in gene expression for *CWC15* [82]. CRISPR-based knockouts have been used to successfully identify embryonic lethals in other species (e.g., [83–85]).

Polygenic Traits

The infinitesimal model, first proposed by Fisher [86], is the foundation of quantitative genetics [87] and provides a mathematical framework based on the idea that phenotypes are influenced

by a large number of loci, each with a small effect. Cole et al. [88] concluded that Fisher's model holds for many traits routinely evaluated in dairy cattle populations. Genomic selection is a special case of marker-assisted selection in which a dense marker set is distributed across the genome [89,90], and the SNP used for prediction may be in linkage with the true causal variants, rather than causal variants themselves. The use of advanced reproductive technologies in conjunction with marker-assisted selection was proposed by Georges and Massey [91] as a way to substantially increase rates of genetic gain, and a cost-effective implementation of a similar scheme recently has been demonstrated [92].

Promotion of alleles by gene editing. In a recent paper, Jenko et al. [49] described a system that they call promotion of alleles by genome editing (PAGE), in which gene editing is used to edit hundreds of alleles to fix favorable alleles, thereby increasing the rate of genetic gain and the asymptote of genetic gain. Inbreeding increases as the number of edits increases, and as the number of animals edited decreases, although the authors suggest that this is not a case for concern because favorable alleles are being fixed rather than deleterious alleles. In the most ambitious scenario considered – 100 QTN edited in each of the top 5 sires – cumulative genetic gains were more than 4 times greater than in the baseline scenario of genomic selection with no editing. A more modest doubling was observed when the 20 loci with the largest effects were edited in all 25 bulls in the population each generation, a much more plausible scenario than editing 100 loci in a single animal. It should be noted that the number of unique loci edited was smaller than the total number edited because the same QTN could be present in many animals. Performing more edits on a smaller number of sires resulted in greater response to selection than performing fewer edits on more bulls when editing resources were limited. However, this also results in substantially greater inbreeding. The authors note that the potential of PAGE to dramatically increase rates of genetic gain is based on the fact that it permits the selection of favorable alleles independently of the haplotypes, chromosomes, and individuals that carry them. Ultimately, PAGE overcomes the barrier represented by the limited number of recombinations that occur during meiosis in gametogenesis.

In an independent study of PAGE, Simianer et al. [93] concluded from their own study of PAGE that realistic gains are almost certain to be much smaller than those predicted by Jenko et al. [49], on the order of 11.6% more than GBLUP, rather than ~400%. Perhaps the most important difference between the two studies is that Jenko et al. [49] assumed that true QTN were known and could be edited directly, while Simianer et al. [93] used ridge regression to estimate SNP effects and edited the markers with the largest effects. In that case, the 20 loci with the largest effect were edited within each sire. Given that true QTN are unknown in our populations, and we are unlikely to identify them without some error, it seems plausible that the initial study of PAGE over-estimated rates of gain that can be expected in practice. These results underscore the importance of assumptions when comparing simulation studies. However, that simply means that PAGE will produce lower rates of gain than originally predicted, not that the approach is without value. The challenge for practitioners will be to balance predicted benefits against the cost of implementation. It could be the case that alternative breeding schemes, such as those using , will be more cost-effective in practice.

Accepting, for the sake of argument, that low-cost gene editing of many alleles in single individual is possible, the loss of genetic variance in the population is of great concern. If the objective of selection is to fix all favorable variants, gene editing can help us do that quickly, and precision edits could be made for the purpose of maintaining variation in the population. This is potentially an important advantage over classical genetic selection that operates on haplotypes, the lengths of which are limited by the rate of recombination. However, if the problem of identifying causal variants is formidable (as discussed below in the section titled "Challenges to the adoption of PAGE", then the challenge of identifying what variants in the population should be maintained because of potential future is more formidable still. Genotype-by-environment effects also may favor some variants in one place or under one management system, but different variants in others. Even if the edited regions of the genome are free of undesirable alleles there is no guarantee that the unmodified parts of the genome will be, and the intense within-family selection driven by genomic selection will only increase. This would result in a loss of variation across the genome, not only in edited regions. Ultimately, dairy breeding programs might then come to resemble swine or poultry programs, in which crosses are made among inbred lines. It also would ultimately favor large breeding companies with the financial resources necessary to produce gene edited animals over individual registered breeders and small genetics firms that currently can compete in the marketplace.

Maintenance of genetic variation. While early research suggested that inbreeding in dairy cattle would decrease under genomic selection [94], it appears to have increased in practice [95,96]. There is a substantial body of literature on the deleterious effects of inbreeding on livestock performance (e.g., [97,98]), and many schemes have been proposed to control levels of inbreeding in both traditional [99] and genomic [100] breeding programs. Recessive and partially recessive deleterious alleles appear to be the principal drivers of inbreeding depression [101], suggesting that genome engineering may be a useful tool for reducing genetic load through targeted editing of known deleterious alleles. Immune gene regions also could be targeted for editing to ensure that low effective population sizes from intensive selection are not accompanied by loss of allelic diversity. Thompson-Crispi et al. [102] found that cell- and antibody-mediated immune responses are under genetic control, and identified several genomic regions [103] that are likely targets for genome engineering. It seems likely that increased homozygosity has harmful effects in some parts of the genome, and neutral or beneficial effects in others. One option for making PAGE schemes more appealing is to include edits for the purpose of increasing heterozygosity in those regions where it is known to be beneficial.

Gene stacking. Gene stacking [104] is the process by which two or more transgenes are accumulated in a single individual, often through intercrossing of transfected lines. While several approaches can be used for this purpose, all are time-consuming and labor-intensive. In plants, where the technique is most commonly used, the accumulation of three or four transgenes in a single line is considered very successful. New tools may increase the efficiency of gene stacking in some species of plants [105], but similar resources are not yet available for use with mammalian genomes. Fischer et al. [106] have recently used gene stacking in

conjunction with gene editing and bacterial- and phage artificial chromosome vectors to produce pigs for xenotransplantation whose genomes contain several transgenes, as well as knockouts. In concept, the use of gene editing to simultaneously modify several loci is simpler than gene stacking, but many technical challenges related to off-target insertions and unintended mutations remain [107,108].

Cole and VanRaden [109] described a conceptually similar approach that could be used to accumulate in one (e.g., *Bos taurus*) individual the 29 autosomes in the population having the highest genetic merit. When haplotypes are sampled at random during gametogenesis there are 2^{29} possible combinations of chromosomes, and there are many more when recombination is considered. Given than haplotypes segregate independently, there is no way to produce animals with a specific set of haplotypes short of crossing completely inbred lines. This challenge, on a smaller scale, also affects gene stacking programs, particularly when genes to be stacked are on different chromosomes and assort independently.

Gene drive. First proposed by Burt [110] as a means of controlling natural populations, such as mosquitoes which transmit malaria, gene drives make use of DNA repair mechanisms to ensure that mutations on one chromosome are copied onto the homologous chromosome. Gonen et al. [111] have suggested that gene drives could be incorporated into livestock breeding programs that include gene editing. The advantage of such approach is that edited alleles would reach fixation more quickly, ensuring homozygosity of the favorable allele in all descendants of the edited individual regardless of the genotype of the other parent. Their simulation found that gene editing produced 1.95 times as much gain as selection alone, gene drives achieved 1.43 times more gain that gene editing alone, and gene drives produced 2.8 times as much gain as selection alone. Allele frequencies reached fixation much faster with either genomic technology than selection alone, although gains from adding gene drives to gene editing were small. The rate of increase in population average inbreeding, already concerning under selection, increased dramatically when gene drives were used only in a small number of top bulls. The broader the portfolio of bulls used, the lower the impact on inbreeding. These results show that gene drives have the potential to amplify the benefits of genome editing in livestock breeding by reducing the time needed to fix favorable alleles. Cumulative genetic gain also is increased because, once the first group of edited alleles is fixed, the resources needed to edit the first group can be allocated to the alleles with the next-smallest effects.

There is considerable concern about the risks associated with using gene drives (e.g., [112]), and regulators may prohibit its use in livestock in the absence of a more compelling argument than increased rates of genetic gain. However, Zentner et al. [113] note that a number of biological mechanisms, such as inbreeding and naturally occurring variation, can interfere with the effectiveness of gene drives, which may limit the appeal and effectiveness of those tools in livestock populations.

Future outlook: opportunities and obstacles

Challenges to the adoption of PAGE

What loci should be edited? A critical issue not addressed by studies to date is that of which alleles should be edited. While this is understandable given that the purpose of the initial work was to demonstrate the utility of the PAGE concept, it must be addressed at the time of implementation. Weller et al. [114] used an *a posteriori* granddaughter design that included 52 grandsire families with 9,178 sons to identify QTL in the US Holstein population, identifying 30 significant QTL. In a follow-up study with 71 grandsire families with 14,246 sons, Wiggans and Weller [115] identified 56 QTL, 29 of which were confirmed from their earlier report. Recently, they used data from the 1000 Bull Genomes Project and other sources to determine concordance between QTL identified from SNP data and quantitative trait nucleotides (QTN) [116]. They found complete or almost-complete concordance only for stature on chromosome 14 and daughter pregnancy rate on chromosome 18. These results underscore the difficulty of identifying the hundreds of editing targets needed for the most effective PAGE strategies. The human genetics community faces similar challenges and has developed a set of guidelines to guide research into causal variants [117] that could be adapted to the needs of livestock research. Given enough time, international efforts such as the Functional Annotation of Animal Genomes project [118] may identify true variants that can be used as targets for editing, but the community is relatively small and constrained by financial limitations, limiting its ability to rapidly make progress.

It is also likely that the problem of identifying loci for editing is of greater magnitude than commonly assumed. Visscher et al. [119] concluded that, “it is the cumulative effect of many loci that underlies susceptibility to disease”, which they reiterated in a subsequent review [120]. Gianola et al. [121] also noted, based on a simulation study, that models commonly used for GWAS do not properly account for linkage disequilibrium, resulting in spurious or misleading results. In a simulation study, Jenko et al. [122] examined the power of using a population of 1 million sequenced animals to identify causal variants, but found that only a small proportion of true variants (2.5–4.8%) were discovered. These results are broadly in agreement with those of VanRaden et al. [123], who found that the addition of sequence variants to SNP used for genomic prediction provided only small gains in reliability because nearby markers already account for the effect of causal variants. Hickey et al. [50] proposed a scheme for testing alleles to differentiate between causal and non-causal alleles based on identification of candidate causal alleles using large-scale GWAS, followed by editing of desirable causal alleles into sire lines for testing in progeny. This allele testing strategy could be used in concert with the allele specific expression approach described by Khansefid et al. [124] to precisely identify true causal variants, which could then be rapidly introgressed into populations using accelerated breeding schemes (e.g., [92,125]).

These results underscore a critical point: gene editing is unlikely to result in substantial improvement of complex traits because 1) we lack the knowledge of direct effects of, and interactions among, individual loci needed to identify targets for editing, 2) our widely used statistical models may not be sufficient for identifying true causal variants, and 3) it seems implausible to assume that simultaneous, side-effect-free editing of hundreds of loci will ever

be feasible. However, gene editing could be useful for the improvement of monogenic traits, either through correction of genetic defects or promotion of desirable alleles, such as polled.

Limits to editing technology. Several technical challenges with gene editing technology must be overcome before PAGE can be implemented. The most notable of these is the development of low-cost tools for multiplexing edits; Hickey et al. [50] argue that this may be the easiest challenge to address, but the difference between 20 and 250 edits is substantial, and there is reason for considerable skepticism that this is an easily solved problem. Recent studies have identified off-target insertions and unintended mutations [107,108] associated with CRISPR editing, raising the possibility that the number of possible simultaneous edits will remain low to avoid the accumulation of uncontrolled changes. In such a case, the effectiveness of PAGE-based strategies will more closely resemble gene stacking (discussed below) or more traditional marker-assisted breeding schemes (e.g., [126]).

Selection limits. Cole and VanRaden [109] have argued that there is no evidence yet that dairy cattle are nearing selection limits, but such limits surely exist. In addition, non-genetic factors such as animal health and feed intake impose limits on phenotypic responses to genetic selection. High-performing animals currently face many challenges, most notably in the transition from pregnancy to lactation, and there will be a point at which it makes more sense to maintain a somewhat larger herd of animals with slightly lower genetic potential than to continue selecting for, e.g., greater fat and protein production.

Unanticipated uses of technology. The success of animal breeding programs depends on good-faith participation by many individuals (e.g., [98]). Technologies such as gene editing are inherently value-neutral and can be used for the mutual benefit of all, as well as for the specific benefit of only one party. For example, if lost-cost, multiplex gene editing is eventually realized then individuals could be edited so that their genome contains the most favorable alleles for each of the markers in the SNP panels used for genomic evaluation, regardless of the animal's true genetic background. This requires that population-specific SNP effects are known, but approximate values can be back-calculated using publicly available genomic breeding values if a an individual has access to a large enough library of genotypes. The resulting animals would receive very high genomic evaluations and would have high apparent value in the marketplace but would not provide the level of genetic improvement suggested by their genomic evaluations. However, if there is no "signature" of the editing process that unambiguously prove that such a genotype resulted from human intervention rather than chance then confidence in the system is undermined to benefit one at the expense of the community. The solution to this problem is probably to develop systems that incentivize desirable behavior, but we should not be blind to the existence of bad actors.

Regulatory considerations

While some genetically modified and gene-edited products recently have reached the U.S. marketplace [128,129], uncertainty about the manner in which gene-edited plant and animal products will be regulated remains a substantial concern [130]. The AquaAdvantage salmon,

genetically engineered for rapid growth, was finally approved for sale following a twenty-year review by federal regulatory agencies [128]. Much of the discussion at the 2016 Large Animal Genetic Engineering Summit focused on the use of gene editing to produce large animal models of human disease (e.g., [131]) rather than modified food animals, possibly in response to an ongoing climate of regulatory uncertainty, although there was more discussion of gene-edited animals at the 2018 conference. It also is unclear if consumers will readily accept the widespread introduction of gene-edited animals in the food chain. Policymakers and regulators are being encouraged to exercise oversight based on the product rather than the process used to generate that product [132], but the Court of Justice of the European Union implicitly rejected this approach when ruling recently that gene-edited crops are subject to the same regulations as conventional genetically modified organisms [133].

Consumer acceptance of gene editing

Many challenges are associated with both genetically engineered and gene-edited animals, some technical and others related to consumer attitudes towards the technology [134,135]. While the tools available for making changes to animals' genomes have increased in capability in recent years, the general public remains concerned about changes made to the genomes of food crops and livestock. The term "genetically modified organism" often is used in discussions of consumer and regulatory affairs, language which unfortunately conflates very different technologies. that term will be used in the following discussion for consistency with the literature discussed, and should be understood to refer to a broad array of technologies that includes both genetic engineering and gene editing.

A recent meta-analysis of the literature on consumer preferences suggests that U.S. respondents have a more favorable view of biotechnologically modified food products than those from Europe, but most consumers are concerned about genetically modified animals [136]. Consumers that are generally opposed to the marketing of genetically modified organisms may moderate those opinions in the presence of another benefit (e.g., increased levels of omega-3 fatty acids in farmed salmon) [137]. Changing consumer attitudes towards technologies may be possible, but the discussion should focus on the benefits rather than the technology [138]. It is difficult to predict how consumers will respond to the idea of dozens or hundreds of simultaneous edits being made to an individual's genome, particularly when current knowledge of interactions among loci is very limited.

Consumers may be more accepting of gene editing in food animals if the technology focus is on animal health and welfare rather than on productivity [139], and there is less objection to the promotion of naturally occurring genetic variants [140,141]. For example, the process of dehorning is traumatic to calves, unpleasant for farmers, and distasteful to consumers (e.g., [142]). Previous studies [143,144] have shown that increasing the frequency of polled animals in the Holstein population is difficult because the frequency of the dominant allele is very low. Carlson et al. [45] have successfully produced polled clones of horned animals using gene editing with no detectable off-target effects, which showed that the technology can be used to rapidly propagate desirable genotypes. Gene editing also has been used to produce

animals with increased resistance to disease [145], including porcine reproductive and respiratory syndrome [146,147] and bovine tuberculosis [54]. Other candidates for gene editing include casein variants that may have beneficial effects on human health [148], the slick locus that is involved in adaptation to hot environments [65], and the *DGAT1* gene which has favorable effects on milk composition [149].

Conclusion/Summary

The rapid development of tools for the precision editing of livestock genomes provides an exciting view of a future in which selection objectives can be rapidly achieved using a combination of advanced reproductive technologies, genomic selection, and genome engineering with low risk of accumulation of harmful genetic defects in the population. However, this future depends on a large body of knowledge that has not yet been generated. We will probably never learn the exact function of every gene in the bovine genome, or the precise genetic mechanism that underlies every genetic defect, not because the problem is insoluble but because it requires human resources and financial capital that are not available to us. The ultimate goal of this work is to use new technology to feed a growing population with fewer inputs, which will depend on gaining the consent of consumers who include animal protein in their diets. It is not enough to assert the safety of these tools, it must be proven with rigorous studies that are openly discussed if regulatory agencies are to be satisfied that the animals produced using these tools are safe for human consumption. As the population in the global south increases there may be a divide in adoption of the technology, with genome engineered food common in some parts of the world and prohibited in others.

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Reference1. García-Ruiz A, Cole JB, VanRaden PM, Wiggans GR, Ruiz-López FJ, Tassell CPV. Changes in genetic selection differentials and generation intervals in US Holstein dairy cattle as a result of genomic selection. Proc Natl Acad Sci. 2016;113: E3995–E4004. doi:10.1073/pnas.1519061113

2. Jonas E, de Koning D-J. Genomic selection needs to be carefully assessed to meet specific requirements in livestock breeding programs. Front Genet. 2015;6. doi:10.3389/fgene.2015.00049

3. Harris DL, Newman S. Breeding for profit: synergism between genetic improvement and livestock production (a review). J Anim Sci. 1994;72: 2178–2200.

4. Crossa J, Pérez-Rodríguez P, Cuevas J, Montesinos-López O, Jarquín D, de los Campos G, et al. Genomic Selection in Plant Breeding: Methods, Models, and Perspectives. Trends Plant Sci. 2017;22: 961–975. doi:10.1016/j.tplants.2017.08.011

5. Bourdon RM. Understanding Animal Breeding. 2nd ed. Englewood Cliffs, New Jersey: Prentice Hall; 1999.

6. Groen AF, Smith C. A stochastic simulation study of the efficiency of marker-assisted introgression in livestock. J Anim Breed Genet. 1995;112: 161–170.

7. Hammer RE, Pursel VG, Jr CER, Wall RJ, Bolt DJ, Ebert KM, et al. Production of transgenic rabbits, sheep and pigs by microinjection. Nature. 1985;315: 680–683. doi:10.1038/315680a0

8. Kues WA, Niemann H. Advances in farm animal transgenesis - ScienceDirect. Prev Vet Med. 2011;102: 146–156. doi:https://doi.org/10.1016/j.prevetmed.2011.04.009

9. Forabosco F, Löhmus M, Rydhmer L, Sundström LF. Genetically modified farm animals and fish in agriculture: A review. Livest Sci. 2013;153: 1–9. doi:10.1016/j.livsci.2013.01.002

10. Wall RJ, Powell AM, Paape MJ, Kerr DE, Bannerman DD, Pursel VG, et al. Genetically enhanced cows resist intramammary *Staphylococcus aureus* infection. Nat Biotechnol. 2005;23: 445–451. doi:10.1038/nbt1078

11. Houdebine LM. Transgenic animal bioreactors. Transgenic Res. 2000;9: 305–320. doi:10.1023/A:1008934912555

12. Xu H-T, Fan B-L, Yu S-Y, Huang Y-H, Zhao Z-H, Lian Z-X, et al. Construct Synthetic Gene Encoding Artificial Spider Dragline Silk Protein and its Expression in Milk of Transgenic Mice. Anim Biotechnol. 2007;18: 1–12. doi:10.1080/10495390601091024

13. Brophy B, Smolenski G, Wheeler T, Wells D, L'Huillier P, Laible G. Cloned transgenic cattle produce milk with higher levels of beta-casein and kappa-casein. NatBiotechnol. 2003; Available: PM:12548290

14. Berkel PHC van, Welling MM, Geerts M, Veen HA van, Ravensbergen B, Salaheddine M, et al. Large scale production of recombinant human lactoferrin in the milk of transgenic cows. Nat Biotechnol. 2002;20: 484–487. doi:10.1038/nbt0502-484

15. Kambadur R, Sharma M, Smith TPL, Bass JJ. Mutations in *myostatin* (*GDF8*) in Double-Muscled Belgian Blue and Piedmontese Cattle. Genome Res. 1997;7: 910–915. doi:10.1101/gr.7.9.910

16. Wu X, Ouyang H, Duan B, Pang D, Zhang L, Yuan T, et al. Production of cloned transgenic cow expressing omega-3 fatty acids. Transgenic Res. 2012;21: 537–543. doi:10.1007/s11248-011-9554-2

17. Blasco A. The role of genetic engineering in livestock production. Livest Sci. 2008;113: 191–201. doi:10.1016/j.livsci.2007.03.012

18. Greger M. Trait selection and welfare of genetically engineered animals in agriculture. J Anim Sci. 2010;88: 811–814. doi:10.2527/jas.2009-2043

19. Eriksson S, Jonas E, Rydhmer L, Röcklinsberg H. Invited review: Breeding and ethical perspectives on genetically modified and genome edited cattle. J Dairy Sci. 2017;0. doi:10.3168/jds.2017-12962

20. Wang M, Sun Z, Yu T, Ding F, Li L, Wang X, et al. Large-scale production of recombinant human lactoferrin from high-expression, marker-free transgenic cloned cows. Sci Rep. 2017;7: 10733. doi:10.1038/s41598-017-11462-z

21. Akar B, Tatara AM, Sutradhar A, Hsiao H-Y, Miller M, Cheng M-H, et al. Large Animal Models of an In Vivo Bioreactor for Engineering Vascularized Bone. Tissue Eng Part B Rev. 2018;24: 317–325. doi:10.1089/ten.teb.2018.0005

22. Doudna JA, Charpentier E. The new frontier of genome engineering with CRISPR-Cas9. Science. 2014;346: 1258096. doi:10.1126/science.1258096

23. Carlson DF, Tan W, Lillico SG, Stverakova D, Proudfoot C, Christian M, et al. Efficient TALEN-mediated gene knockout in livestock. Proc Natl Acad Sci. 2012;109: 17382–17387. doi:10.1073/pnas.1211446109

24. Carroll D. Genome Engineering With Zinc-Finger Nucleases. Genetics. 2011;188: 773–782. doi:10.1534/genetics.111.131433

25. Yum S-Y, Youn K-Y, Choi W-J, Jang G. Development of genome engineering technologies in cattle: from random to specific. J Anim Sci Biotechnol. 2018;9: 16. doi:10.1186/s40104-018-0232-6

26. Gaj T, Gersbach CA, Barbas CF. ZFN, TALEN and CRISPR/Cas-based methods for genome engineering. Trends Biotechnol. 2013;31: 397–405. doi:10.1016/j.tibtech.2013.04.004

27. Sander JD, Joung JK. CRISPR-Cas systems for editing, regulating and targeting genomes. Nat Biotechnol. 2014;32: 347–355. doi:10.1038/nbt.2842

28. Van Eenennaam AL. Genetic modification of food animals. Curr Opin Biotechnol. 2017;44: 27–34. doi:10.1016/j.copbio.2016.10.007

29. Shanthalingam S, Tibary A, Beever JE, Kasinathan P, Brown WC, Srikumaran S. Precise gene editing paves the way for derivation of *Mannheimia haemolytica* leukotoxin-resistant cattle. Proc Natl Acad Sci. 2016;113: 13186–13190. doi:10.1073/pnas.1613428113

30. Lillico SG, Proudfoot C, Carlson DF, Stverakova D, Neil C, Blain C, et al. Live pigs produced from genome edited zygotes. Sci Rep. 2013;3. doi:10.1038/srep02847

31. Li T, Huang S, Jiang WZ, Wright D, Spalding MH, Weeks DP, et al. TAL nucleases (TALNs): hybrid proteins composed of TAL effectors and FokI DNA-cleavage domain. Nucleic Acids Res. 2011;39: 359–372. doi:10.1093/nar/gkq704

32. Carlson DF, Lancto CA, Zang B, Kim E-S, Walton M, Oldeschulte D, et al. Production of hornless dairy cattle from genome-edited cell lines. Nat Biotechnol. 2016;34: 479–481. doi:10.1038/nbt.3560

33. Park TS, Lee HJ, Kim KH, Kim J-S, Han JY. Targeted gene knockout in chickens mediated by TALENs. Proc Natl Acad Sci. 2014;111: 12716–12721. doi:10.1073/pnas.1410555111

34. Jinek M, Chylinski K, Fonfara I, Hauer M, Doudna JA, Charpentier E. A Programmable Dual-RNA-Guided DNA Endonuclease in Adaptive Bacterial Immunity. Science. 2012;337: 816–821. doi:10.1126/science.1225829

35. Zetsche B, Gootenberg JS, Abudayyeh OO, Slaymaker IM, Makarova KS, Essletzbichler P, et al. Cpf1 Is a Single RNA-Guided Endonuclease of a Class 2 CRISPR-Cas System. Cell. 2015;163: 759–771. doi:10.1016/j.cell.2015.09.038

36. Ran FA, Cong L, Yan WX, Scott DA, Gootenberg JS, Kriz AJ, et al. In vivo genome editing using Staphylococcus aureus Cas9. Nature. 2015;520: 186–191. doi:10.1038/nature14299

37. Kim E, Koo T, Park SW, Kim D, Kim K, Cho H-Y, et al. In vivo genome editing with a small Cas9 orthologue derived from Campylobacter jejuni. Nat Commun. 2017;8: 14500. doi:10.1038/ncomms14500

38. Kleinstiver BP, Prew MS, Tsai SQ, Topkar VV, Nguyen NT, Zheng Z, et al. Engineered CRISPR-Cas9 nucleases with altered PAM specificities. Nature. 2015;523: 481–485. doi:10.1038/nature14592

39. Kleinstiver BP, Prew MS, Tsai SQ, Nguyen NT, Topkar VV, Zheng Z, et al. Broadening the targeting range of Staphylococcus aureus CRISPR-Cas9 by modifying PAM recognition. Nat Biotechnol. 2015;33: 1293–1298. doi:10.1038/nbt.3404

40. Ding Q, Regan SN, Xia Y, Oostrom LA, Cowan CA, Musunuru K. Enhanced efficiency of human pluripotent stem cell genome editing through replacing TALENs with CRISPRs. Cell Stem Cell. 2013;12: 393–394. doi:10.1016/j.stem.2013.03.006

41. Wang H, Yang H, Shivalila CS, Dawlaty MM, Cheng AW, Zhang F, et al. One-Step Generation of Mice Carrying Mutations in Multiple Genes by CRISPR/Cas-Mediated Genome Engineering. Cell. 2013;153: 910–918. doi:10.1016/j.cell.2013.04.025

42. Gao Y, Wu H, Wang Y, Liu X, Chen L, Li Q, et al. Single Cas9 nickase induced generation of NRAMP1 knockin cattle with reduced off-target effects. Genome Biol. 2017;18: 13. doi:10.1186/s13059-016-1144-4

43. Ni W, Qiao J, Hu S, Zhao X, Regouski M, Yang M, et al. Efficient gene knockout in goats using CRISPR/Cas9 system. PloS One. 2014;9: e106718. doi:10.1371/journal.pone.0106718

44. Park K-E, Kaucher AV, Powell A, Waqas MS, Sandmaier SES, Oatley MJ, et al. Generation of germline ablated male pigs by CRISPR/Cas9 editing of the *NANOS2* gene. Sci Rep. 2017;7: 40176. doi:10.1038/srep40176

45. Maeder ML, Gersbach CA. Genome-editing Technologies for Gene and Cell Therapy. Mol Ther. 2016;24: 430–446. doi:10.1038/mt.2016.10

46. Cole JB. Management of Mendelian Traits in Breeding Programs by Gene Editing: A Simulation Study. bioRxiv. 2017; 116459. doi:10.1101/116459

47. Johnsson M, Gaynor RC, Jenko J, Gorjanc G, Koning D-J de, Hickey JM. Removal of alleles by genome editing — RAGE against the deleterious load. bioRxiv. 2018; 335497. doi:10.1101/335497

48. Bastiaansen JWM, Bovenhuis H, Groenen MAM, Megens H-J, Mulder HA. The impact of genome editing on the introduction of monogenic traits in livestock. Genet Sel Evol. 2018;50: 18. doi:10.1186/s12711-018-0389-7

49. Jenko J, Gorjanc G, Cleveland MA, Varshney RK, Whitelaw CBA, Woolliams JA, et al. Potential of promotion of alleles by genome editing to improve quantitative traits in livestock breeding programs. Genet Sel Evol. 2015;47: 1–14. doi:10.1186/s12711-015-0135-3

50. Hickey JM, Bruce C, Whitelaw A, Gorjanc G. Promotion of alleles by genome editing in livestock breeding programmes. J Anim Breed Genet. 2016;133: 83–84. doi:10.1111/jbg.12206

51. Yu S, Luo J, Song Z, Ding F, Dai Y, Li N. Highly efficient modification of *beta-lactoglobulin (BLG)* gene via zinc-finger nucleases in cattle. Cell Res. 2011;21: 1638–1640. doi:10.1038/cr.2011.153

735 52. Wei J, Wagner S, Maclean P, Brophy B, Cole S, Smolenski G, et al. Cattle with a precise,
736 zygote-mediated deletion safely eliminate the major milk allergen beta-lactoglobulin. Sci
737 Rep. 2018;8: 7661. doi:10.1038/s41598-018-25654-8

738 53. Liu X, Wang Y, Guo W, Chang B, Liu J, Guo Z, et al. Zinc-finger nickase-mediated insertion of
739 the lysostaphin gene into the beta-casein locus in cloned cows. Nat Commun. 2013;4: 2565.
740 doi:10.1038/ncomms3565

741 54. Liu X, Wang Y, Tian Y, Yu Y, Gao M, Hu G, et al. Generation of mastitis resistance in cows by
742 targeting human lysozyme gene to β-casein locus using zinc-finger nucleases. Proc R Soc B.
743 2014;281: 20133368. doi:10.1098/rspb.2013.3368

744 55. Luo J, Song Z, Yu S, Cui D, Wang B, Ding F, et al. Efficient Generation of Myostatin (MSTN)
745 Biallelic Mutations in Cattle Using Zinc Finger Nucleases. PLOS ONE. 2014;9: e95225.
746 doi:10.1371/journal.pone.0095225

747 56. Proudfoot C, Carlson DF, Huddart R, Long CR, Pryor JH, King TJ, et al. Genome edited sheep
748 and cattle. Transgenic Res. 2015;24: 147–153. doi:10.1007/s11248-014-9832-x

749 57. Tan W, Carlson DF, Lancto CA, Garbe JR, Webster DA, Hackett PB, et al. Efficient nonmeiotic
750 allele introgression in livestock using custom endonucleases. Proc Natl Acad Sci. 2013;110:
751 16526–16531. doi:10.1073/pnas.1310478110

752 58. Bevacqua RJ, Fernandez-Martín R, Savy V, Canel NG, Gismondi MI, Kues WA, et al. Efficient
753 edition of the bovine PRNP prion gene in somatic cells and IVF embryos using the
754 CRISPR/Cas9 system. Theriogenology. 2016;86: 1886-1896.e1.
755 doi:10.1016/j.theriogenology.2016.06.010

756 59. Choi W, Kim E, Yum S-Y, Lee C, Lee J, Moon J, et al. Efficient PRNP deletion in bovine
757 genome using gene-editing technologies in bovine cells. Prion. 2015;9: 278–291.
758 doi:10.1080/19336896.2015.1071459

759 60. Wu H, Wang Y, Zhang Y, Yang M, Lv J, Liu J, et al. TALE nickase-mediated SP110 knockin
760 endows cattle with increased resistance to tuberculosis. Proc Natl Acad Sci. 2015;
761 201421587. doi:10.1073/pnas.1421587112

762 61. Mendel G. Experiments in plant hybridization. Cambridge, MA: Harvard University Press;
763 1965.

764 62. Medugorac I, Seichter D, Graf A, Russ I, Blum H, Göpel KH, et al. Bovine polledness – an
765 autosomal dominant trait with allelic heterogeneity. PLOS ONE. 2012;7: e39477.
766 doi:10.1371/journal.pone.0039477

767 63. Mueller ML, Cole JB, Sonstegard TS, Eenennaam ALV. Simulation of Introgression of the
768 Polled Allele into the Holstein Breed via Conventional Breeding versus Gene Editing.
769 Proceedings of 11th World Congress of Genetics Applied to Livestock Production. Auckland,

770 NZ; 2018. Available: http://www.wcgalp.org/proceedings/2018/simulation-introgression-
771 polled-allele-holstein-breed-conventional-breeding-versus

772 64. Hai T, Teng F, Guo R, Li W, Zhou Q. One-step generation of knockout pigs by zygote
773 injection of CRISPR/Cas system. Cell Res. 2014;24: 372–375. doi:10.1038/cr.2014.11

774 65. Dikmen S, Khan FA, Huson HJ, Sonstegard TS, Moss JI, Dahl GE, et al. The SLICK hair locus
775 derived from Senepol cattle confers thermotolerance to intensively managed lactating
776 Holstein cows. J Dairy Sci. 2014;97: 5508–5520. doi:10.3168/jds.2014-8087

777 66. Littlejohn MD, Henty KM, Tiplady K, Johnson T, Harland C, Lopdell T, et al. Functionally
778 reciprocal mutations of the prolactin signalling pathway define hairy and slick cattle. Nat
779 Commun. 2014;5. doi:10.1038/ncomms6861

780 67. Porto-Neto LR, Bickhart DM, Landaeta-Hernandez AJ, Utsunomiya YT, Pagan M, Jimenez E,
781 et al. Convergent Evolution of Slick Coat in Cattle through Truncation Mutations in the
782 Prolactin Receptor. Front Genet. 2018;9. doi:10.3389/fgene.2018.00057

783 68. Nicholas FW. Online Mendelian Inheritance in Animals (OMIA): a comparative
784 knowledgebase of genetic disorders and other familial traits in non-laboratory animals.
785 Nucleic Acids Res. 2003;31: 275–277.

786 69. Robertson A, Rendel JM. The use of progeny testing with artificial insemination in dairy
787 cattle. J Genet. 1950;50: 21–31. doi:10.1007/BF02986791

788 70. Shuster DE, Kehrli ME, Ackermann MR, Gilbert RO. Identification and prevalence of a
789 genetic defect that causes leukocyte adhesion deficiency in Holstein cattle. Proc Natl Acad
790 Sci U S A. 1992;89: 9225–9229.

791 71. Agerholm JS, Bendixen C, Andersen O, Arnbjerg J. Complex vertebral malformation in
792 Holstein calves. JVetDiagnInvest. 2001;13: 283–289.

793 72. Shanks RD, Dombrowski DB, Harpestad GW, Robinson JL. Inheritance of UMP synthase in
794 dairy cattle. J Hered. 1984;75: 337–340.

795 73. Sonstegard TS, Cole JB, VanRaden PM, Van Tassell CP, Null DJ, Schroeder SG, et al.
796 Identification of a Nonsense Mutation in CWC15 Associated with Decreased Reproductive
797 Efficiency in Jersey Cattle. PLoS ONE. 2013;8: e54872. doi:10.1371/journal.pone.0054872

798 74. VanRaden PM, Olson KM, Null DJ, Hutchison JL. Harmful recessive effects on fertility
799 detected by absence of homozygous haplotypes. J Dairy Sci. 2011;94: 6153–6161.
800 doi:10.3168/jds.2011-4624

801 75. Charlier C, Coppieters W, Rollin F, Desmecht D, Agerholm JS, Cambisano N, et al. Highly
802 effective SNP-based association mapping and management of recessive defects in livestock.
803 Nat Genet. 2008;40: 449–454.

804 76. Hoff JL, Decker JE, Schnabel RD, Taylor JF. Candidate lethal haplotypes and causal mutations
805 in Angus cattle. BMC Genomics. 2017;18: 799. doi:10.1186/s12864-017-4196-2

806 77. Schwarzenbacher H, Burgstaller J, Seefried FR, Wurmser C, Hilbe M, Jung S, et al. A
807 missense mutation in TUBD1 is associated with high juvenile mortality in Braunvieh and
808 Fleckvieh cattle. bioRxiv. 2016; 041921. doi:10.1101/041921

809 78. MacArthur DG, Balasubramanian S, Frankish A, Huang N, Morris J, Walter K, et al. A
810 Systematic Survey of Loss-of-Function Variants in Human Protein-Coding Genes. Science.
811 2012;335: 823–828. doi:10.1126/science.1215040

812 79. Ma H, Marti-Gutierrez N, Park S-W, Wu J, Lee Y, Suzuki K, et al. Correction of a pathogenic
813 gene mutation in human embryos. Nature. 2017;548: 413–419. doi:10.1038/nature23305

814 80. Van Eeneenaam, A, Kinghorn, B. Use of Mate Selection Software to Manage Lethal
815 Recessive Conditions in Livestock Populations. Proceedings of 10th World Congress of
816 Genetics Applied to Livestock Production. Vancouver, BC, Canada; 2014. Available:
817 https://www.asas.org/docs/default-source/wcgalp-
818 posters/408_paper_9819_manuscript_1027_0.pdf?sfvrsn=2

819 81. Fisher P, Hyndman D, Bixley M, Oback F, Popovic L, McGowan L, et al. Potential for genomic
820 selection of bovine embryos. Proc N Z Soc Anim Prod. 2012;72: 156–158.

821 82. Ortega MS, Cole JB, Sonstegard TS, Hansen PJ. Changes in Expression of Genes Associated
822 with Genetic Variation in Pre-Implantation Development of the Bovine Embryo. Reprod
823 Fertil Dev. 2014;26: 175–175. doi:10.1071/RDv26n1Ab123

824 83. Shao X, Tan C, Chen D, Wang Y-L. Placental defects are involved in most gene mutations
825 that cause embryonic and fetal death. Biol Reprod. doi:10.1093/biolre/ioy080

826 84. Tani H, Ohnishi S, Shitara H, Mito T, Yamaguchi M, Yonekawa H, et al. Mice deficient in the
827 Shmt2 gene have mitochondrial respiration defects and are embryonic lethal. Sci Rep.
828 2018;8: 425. doi:10.1038/s41598-017-18828-3

829 85. Daigneault BW, Rajput S, Smith GW, Ross PJ. Embryonic POU5F1 is Required for Expanded
830 Bovine Blastocyst Formation. Sci Rep. 2018;8: 7753. doi:10.1038/s41598-018-25964-x

831 86. Fisher RA. The Correlation between Relatives on the Supposition of Mendelian Inheritance.
832 Earth Environ Sci Trans R Soc Edinb. 1919;52: 399–433. doi:10.1017/S0080456800012163

833 87. Falconer DS, MacKay FC. Introduction to quantitative genetics. Longman; 1996.

834 88. Cole JB, VanRaden PM, O'Connell JR, Van Tassell CP, Sonstegard TS, Schnabel RD, et al.
835 Distribution and location of genetic effects for dairy traits. J Dairy Sci. 2009;92: 2931–2946.
836 doi:10.3168/jds.2008-1762

89. Nejati-Javaremi A, Smith C, Gibson JP. Effect of total allelic relationship on accuracy of evaluation and response to selection. J Anim Sci. 1997;75: 1738–1745.

90. Meuwissen TH. Genomic selection: marker assisted selection on a genome wide scale. J Anim Breed Genet. 2007;124: 321–322.

91. Georges M, Massey JM. Velogenetics, or the synergistic use of marker assisted selection and germ-line manipulation. Theriogenology. 1991;35: 151–159. doi:10.1016/0093-691X(91)90154-6

92. Kasinathan P, Wei H, Xiang T, Molina JA, Metzger J, Broek D, et al. Acceleration of genetic gain in cattle by reduction of generation interval. Sci Rep. 2015;5: srep08674. doi:10.1038/srep08674

93. Simianer H, Pook T, Schlather M. Turning the PAGE – the potential of genome editing in breeding for complex traits revisited. 2018.

94. Daetwyler HD, Villanueva B, Bijma P, Woolliams JA. Inbreeding in genome-wide selection. J Anim Breed Genet. 2007;124: 369–376.

95. Forutan M, Mahyari SA, Baes C, Melzer N, Schenkel FS, Sargolzaei M. Inbreeding and runs of homozygosity before and after genomic selection in North American Holstein cattle. BMC Genomics. 2018;19: 98. doi:10.1186/s12864-018-4453-z

96. Howard JT, Pryce JE, Baes C, Maltecca C. Invited review: Inbreeding in the genomics era: Inbreeding, inbreeding depression, and management of genomic variability. J Dairy Sci. 2017;100: 6009–6024. doi:10.3168/jds.2017-12787

97. Leroy G. Inbreeding depression in livestock species: review and meta-analysis. Anim Genet. 2014; n/a-n/a. doi:10.1111/age.12178

98. Smith LA, Cassell BG, Pearson RE. The effects of inbreeding on the lifetime performance of dairy cattle. J Dairy Sci. 1998;81: 2729–2737.

99. Weigel KA. Controlling Inbreeding in Modern Breeding Programs. J Dairy Sci. 2001;84: E177–E184. doi:10.3168/jds.S0022-0302(01)70213-5

100. Sonesson AK, Woolliams JA, Meuwissen TH. Genomic selection requires genomic control of inbreeding. Genet Sel Evol. 2012;44: 27. doi:10.1186/1297-9686-44-27

101. Charlesworth B, Charlesworth D. The genetic basis of inbreeding depression. Genet Res. 1999;74: 329–340.

102. Thompson-Crispi KA, Sewalem A, Miglior F, Mallard BA. Genetic parameters of adaptive immune response traits in Canadian Holsteins. J Dairy Sci. 2012;95: 401–409. doi:10.3168/jds.2011-4452

103. Thompson-Crispi KA, Sargolzaei M, Ventura R, Abo-Ismail M, Miglior F, Schenkel F, et al. A genome-wide association study of immune response traits in Canadian Holstein cattle. BMC Genomics. 2014;15: 559. doi:10.1186/1471-2164-15-559

104. Halpin C. Gene stacking in transgenic plants – the challenge for 21st century plant biotechnology. Plant Biotechnol J. 2005;3: 141–155. doi:10.1111/j.1467-7652.2004.00113.x

105. Collier R, Thomson JG, Thilmony R. A versatile and robust Agrobacterium-based gene stacking system generates high-quality transgenic Arabidopsis plants. Plant J. 2018;95: 573–583. doi:10.1111/tpj.13992

106. Fischer K, Kraner-Scheiber S, Petersen B, Rieblinger B, Buermann A, Flisikowska T, et al. Efficient production of multi-modified pigs for xenotransplantation by 'combineering', gene stacking and gene editing. Sci Rep. 2016;6: 29081. doi:10.1038/srep29081

107. Ledford H. CRISPR gene editing produces unwanted DNA deletions. In: Nature [Internet]. 16 Jul 2018 [cited 3 Aug 2018]. doi:10.1038/d41586-018-05736-3

108. Schaefer KA, Wu W-H, Colgan DF, Tsang SH, Bassuk AG, Mahajan VB. Unexpected mutations after CRISPR-Cas9 editing in vivo. Nat Methods. 2017;14: 547–548. doi:10.1038/nmeth.4293

109. Cole JB, VanRaden PM. Use of haplotypes to estimate Mendelian sampling effects and selection limits. J Anim Breed Genet. 2011;128: 446–455. doi:10.1111/j.1439-0388.2011.00922.x

110. Burt A. Site-specific selfish genes as tools for the control and genetic engineering of natural populations. Proc R Soc Lond B Biol Sci. 2003;270: 921–928. doi:10.1098/rspb.2002.2319

111. Gonen S, Jenko J, Gorjanc G, Mileham AJ, Whitelaw CBA, Hickey JM. Potential of gene drives with genome editing to increase genetic gain in livestock breeding programs. Genet Sel Evol. 2017;49: 3. doi:10.1186/s12711-016-0280-3

112. Oye KA, Esvelt K, Appleton E, Catteruccia F, Church G, Kuiken T, et al. Regulating gene drives. Science. 2014;345: 626–628. doi:10.1126/science.1254287

113. Zentner GE, Wade MJ. The promise and peril of CRISPR gene drives. BioEssays. 2017;39: 1700109. doi:10.1002/bies.201700109

114. Weller JI, Cole JB, VanRaden PM, Wiggans GR. Application of the a posteriori granddaughter design to the Holstein genome. animal. 2014;8: 511–519. doi:10.1017/S1751731114000111

115. Wiggans G, Weller JI. Revisiting the "a posteriori" granddaughter design. Interbull Bull. 2015;

116. Weller JI, Bickhart DM, Wiggans GR, Tooker ME, O'Connell JR, Jiang J, et al. Determination of quantitative trait nucleotides by concordance analysis between quantitative trait loci and marker genotypes of US Holsteins. J Dairy Sci. 2018;0. doi:10.3168/jds.2018-14816

117. MacArthur DG, Manolio TA, Dimmock DP, Rehm HL, Shendure J, Abecasis GR, et al. Guidelines for investigating causality of sequence variants in human disease. Nature. 2014;508: 469–476. doi:10.1038/nature13127

118. Andersson L, Archibald AL, Bottema CD, Brauning R, Burgess SC, Burt DW, et al. Coordinated international action to accelerate genome-to-phenome with FAANG, the Functional Annotation of Animal Genomes project. Genome Biol. 2015;16: 57. doi:10.1186/s13059-015-0622-4

119. Visscher PM, Brown MA, McCarthy MI, Yang J. Five Years of GWAS Discovery. Am J Hum Genet. 2012;90: 7–24. doi:10.1016/j.ajhg.2011.11.029

120. Visscher PM, Wray NR, Zhang Q, Sklar P, McCarthy MI, Brown MA, et al. 10 Years of GWAS Discovery: Biology, Function, and Translation. Am J Hum Genet. 2017;101: 5–22. doi:10.1016/j.ajhg.2017.06.005

121. Gianola D, Hospital F, Verrier E. Contribution of an additive locus to genetic variance when inheritance is multi-factorial with implications on interpretation of GWAS. Theor Appl Genet. 2013;126: 1457–1472. doi:10.1007/s00122-013-2064-2

122. Jenko J, Whalen A, Gaynor R, Dadousis C, Gorjanc G, Hickey J. Identification of causal variants using one million individuals with whole–genome sequence information. Proc World Congr Genet Appl Livest Prod. 2018;Methods and Tools-GWAS: 591.

123. VanRaden PM, Tooker ME, O'Connell JR, Cole JB, Bickhart DM. Selecting sequence variants to improve genomic predictions for dairy cattle. Genet Sel Evol. 2017;49: 32. doi:10.1186/s12711-017-0307-4

124. Khansefid M, Pryce JE, Bolormaa S, Chen Y, Millen CA, Chamberlain AJ, et al. Comparing allele specific expression and local expression quantitative trait loci and the influence of gene expression on complex trait variation in cattle. BMC Genomics. 2018;19: 793. doi:10.1186/s12864-018-5181-0

125. Goszczynski DE, Cheng H, Demyda-Peyrás S, Medrano JF, Wu J, Ross PJ. In vitro breeding: application of embryonic stem cells to animal production†. Biol Reprod. 2018; doi:10.1093/biolre/ioy256

126. Dekkers JCM. Commercial application of marker- and gene-assisted selection in livestock: Strategies and lessons. J Anim Sci. 2004;82: E313–E328. doi:10.2527/2004.8213_supplE313x

127. Lush JL. Animal Breeding Plans. 2nd ed. Ames, IA: Iowa State College Press; 1947.

128. Ledford H. Salmon approval heralds rethink of transgenic animals. Nat News. 2015;527: 417. doi:10.1038/527417a

129. Waltz E. Gene-edited CRISPR mushroom escapes US regulation. Nat News. 2016;532: 293. doi:10.1038/nature.2016.19754

130. Maxmen A. Gene-edited animals face US regulatory crackdown. Nat News. 2017; doi:10.1038/nature.2017.21331

131. Whitelaw CBA, Sheets TP, Lillico SG, Telugu BP. Engineering large animal models of human disease. J Pathol. 2016;238: 247–256. doi:10.1002/path.4648

132. Carroll D, Van Eenennaam AL, Taylor JF, Seger J, Voytas DF. Regulate genome-edited products, not genome editing itself. Nat Biotechnol. 2016;34: 477–479. doi:10.1038/nbt.3566

133. Callaway E. CRISPR plants now subject to tough GM laws in European Union. In: Nature [Internet]. 25 Jul 2018 [cited 10 Aug 2018]. doi:10.1038/d41586-018-05814-6

134. Tizard M, Hallerman E, Fahrenkrug S, Newell-McGloughlin M, Gibson J, Loos F de, et al. Strategies to enable the adoption of animal biotechnology to sustainably improve global food safety and security. Transgenic Res. 2016;25: 575–595. doi:10.1007/s11248-016-9965-1

135. Zhang C, Wohlhueter R, Zhang H. Genetically modified foods: A critical review of their promise and problems. Food Sci Hum Wellness. 2016;5: 116–123. doi:10.1016/j.fshw.2016.04.002

136. Hess S, Lagerkvist CJ, Redekop W, Pakseresht A. Consumers' evaluation of biotechnologically modified food products: new evidence from a meta-survey. Eur Rev Agric Econ. 2016;43: 703–736. doi:10.1093/erae/jbw011

137. Mather D, Vikan R, Knight J. Marketplace response to GM animal products. Nat Biotechnol. 2016;34: 236–238. doi:10.1038/nbt.3494

138. Ishii T, Araki M. Consumer acceptance of food crops developed by genome editing. Plant Cell Rep. 2016;35: 1507–1518. doi:10.1007/s00299-016-1974-2

139. Frewer LJ, Kleter GA, Brennan M, Coles D, Fischer ARH, Houdebine LM, et al. Genetically modified animals from life-science, socio-economic and ethical perspectives: examining issues in an EU policy context. New Biotechnol. 2013;30: 447–460. doi:10.1016/j.nbt.2013.03.010

140. Croney C, Muir W, Ni J-Q, Widmar NO, Varner G. An Overview of Engineering Approaches to Improving Agricultural Animal Welfare. J Agric Environ Ethics. 2018;31: 143–159. doi:10.1007/s10806-018-9716-9

141. Schultz-Bergin M. Is CRISPR an Ethical Game Changer? J Agric Environ Ethics. 2018;31: 219–238. doi:10.1007/s10806-018-9721-z

142. Thompson NM, Widmar NO, Schutz MM, Cole JB, Wolf CA. Economic considerations of breeding for polled dairy cows versus dehorning in the United States. J Dairy Sci. 2017;100: 4941–4952. doi:10.3168/jds.2016-12099

143. Cole JB. A simple strategy for managing many recessive disorders in a dairy cattle breeding program. Genet Sel Evol. 2015;47: 94. doi:10.1186/s12711-015-0174-9

144. Spurlock DM, Stock ML, Coetzee JF. The impact of 3 strategies for incorporating polled genetics into a dairy cattle breeding program on the overall herd genetic merit. J Dairy Sci. 2014;97: 5265–5274. doi:10.3168/jds.2013-7746

145. Plastow GS. Genomics to benefit livestock production: improving animal health. Rev Bras Zootec. 2016;45: 349–354. doi:10.1590/S1806-92902016000600010

146. Whitworth KM, Rowland RRR, Ewen CL, Trible BR, Kerrigan MA, Cino-Ozuna AG, et al. Gene-edited pigs are protected from porcine reproductive and respiratory syndrome virus. Nat Biotechnol. 2016;34: 20–22. doi:10.1038/nbt.3434

147. Burkard C, Lillico SG, Reid E, Jackson B, Mileham AJ, Ait-Ali T, et al. Precision engineering for PRRSV resistance in pigs: Macrophages from genome edited pigs lacking CD163 SRCR5 domain are fully resistant to both PRRSV genotypes while maintaining biological function. PLOS Pathog. 2017;13: e1006206. doi:10.1371/journal.ppat.1006206

148. Caroli AM, Chessa S, Erhardt GJ. Invited review: Milk protein polymorphisms in cattle: Effect on animal breeding and human nutrition. J Dairy Sci. 2009;92: 5335–5352. doi:10.3168/jds.2009-2461

149. Grisart B, Farnir F, Karim L, Cambisano N, Kim JJ, Kvasz A, et al. Genetic and functional confirmation of the causality of the DGAT1 K232A quantitative trait nucleotide in affecting milk yield and composition. 2004. pp. 2398–2403. Available: PM:14983021