

## Identification and Management of Loss of Function Alleles<sup>1</sup>

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### Abstract

This paper defines some of the scientific terms used when discussing genetic conditions, and reviews the genetic implications and consequences of mating individuals with various genotypes. Many genetic defects are recessive, and the reason for this is that mutant alleles often render the resulting protein nonfunctional. These are called “loss of function” alleles. In many cases if an individual inherits a functional allele from one parent, there is no deleterious phenotype associated with inheriting the loss of function mutant allele from the other parent. As such, a heterozygous “*Aa*” (*A* symbolizes the dominant functional allele, *a* the recessive loss of function allele) animal, or carrier, appears normal. Because carriers appear normal, newly created recessive alleles can increase in frequency in a population more easily than dominant or additive alleles. There is an obvious connection between inbreeding and homozygosity. The main purpose of inbreeding is to make animals more uniform and homozygous for superior genes, however deleterious allelic variants also become homozygous at the same time, unless such variants are lethal in which case there is obvious natural selection against them. With inbreeding, widely used ancestors contribute alleles on both the male and female sides of an animal’s pedigree. It is for this reason that deleterious autosomal recessive alleles are often identified in the descendants of widely used sire lines. It is not because sire’s with excellent genetic merit carry more deleterious alleles, it is because such sires are more likely to be represented on both sides of a pedigree opening up the possibility that an animal will inherit a deleterious allele from both its sire and dam. With the advent of genomic sequencing technologies, our understanding of the prevalence of autosomal recessive conditions has advanced. Given that the average human carries approximately 2,000 deleterious autosomal recessive variants and a similar number is likely to be found in cattle, overtly avoiding the mating of any carrier animals is going to become increasingly unworkable as more deleterious autosomal recessive variants are identified. Management of recessive conditions has to be balanced with other important issues such as the management of trait merit, genetic diversity, other genetic defects, genome-wide inbreeding, logistical constraints and costs. It is likely that decision support software will be required to facilitate the management of this information. Such software will provide an approach to make judicious use of carrier bulls with superior genetic merit while reducing the risk of generating affected calves and strategically working to slowly eradicate the undesirable alleles from the population. While precluding all matings between carrier animals may not be possible, avoiding matings between animals that carry identical alleles is achievable. There is a need to optimize the matings that involve carrier animals in accordance with their genetic merit and actual genotype for undesirable alleles rather than prohibiting their use entirely, and this can best be accomplished using mate selection software.

### What is a loss of function allele?

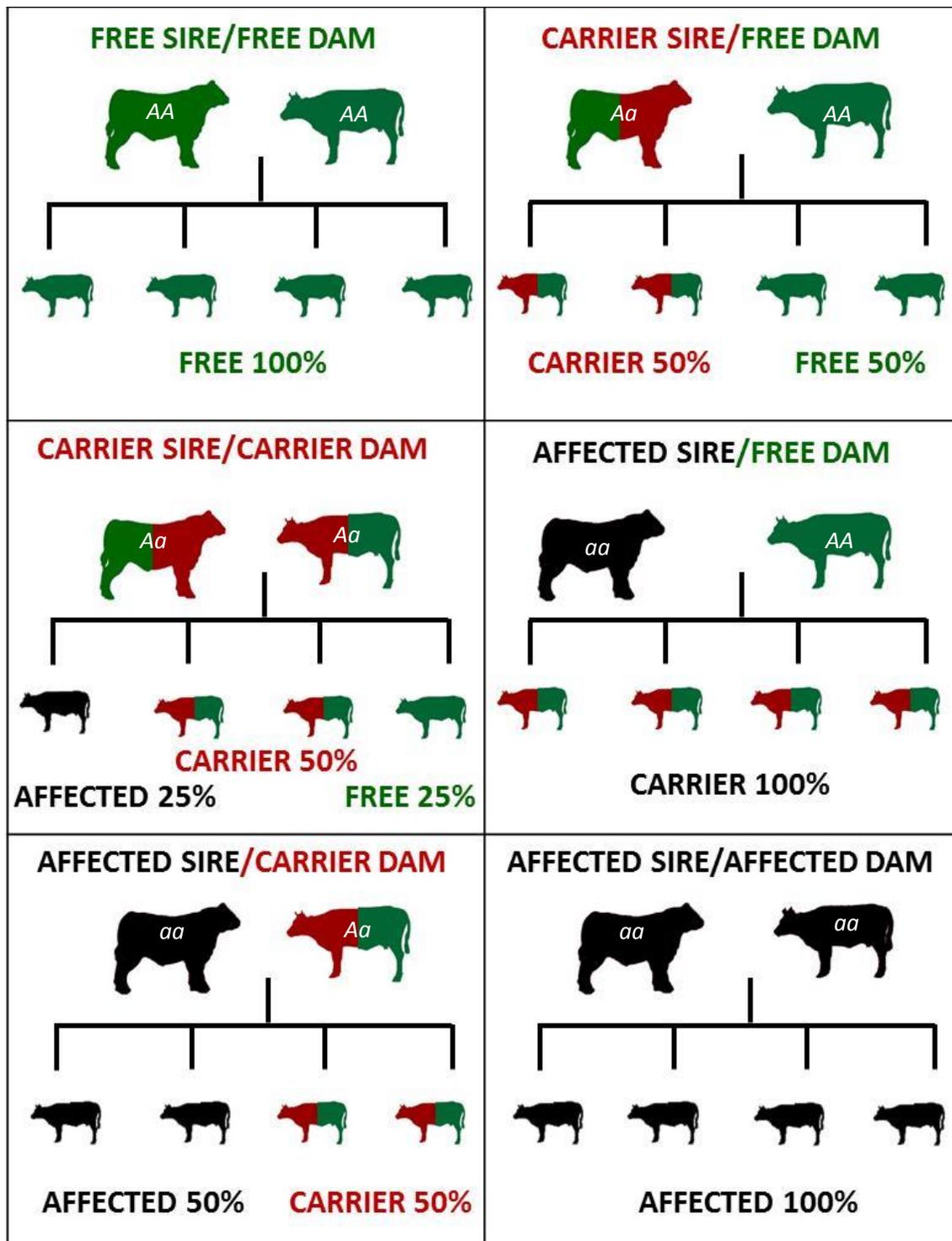
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**Chromosomes** inherited from parents determine an animal's genetic make-up. Chromosomes come in pairs; one chromosome from each pair is inherited from an individual's sire and the other chromosome is inherited from its dam. There are thousands of genes on each chromosome. **Genes** are the basic units of inheritance and they comprise distinct sequences of DNA (A's, T's, C's and G's) that contain all of the instructions for making proteins. It is common for the DNA sequence that makes up a gene or "locus" to differ between individuals. These alternative DNA sequences or forms of a gene are called **alleles**, and they can result in differences in the amount or sequence of protein being produced by that gene among different individual animals. A single nucleotide polymorphism, or **SNP** (pronounced "snip"), occurs when alleles differ from each other by the sequence of only a single nucleotide base pair (i.e., one of the chromosome pairs has a C and the other a G at a particular position) which may lie in a gene or in the DNA that separates the genes on a chromosome (called non-coding DNA).

A **genetic defect** is produced by a mutation that results in an allele with an undesirable **phenotype** (i.e., disease or trait). Some mutations result in gross hereditary defects such as abnormalities in the skeleton, body form, or body functions. Others are associated with a phenotype that may be advantageous in some situations and disadvantageous in others (e.g., the presence or absence of horns). **Homozygous** is a term used to refer to an animal that carries two identical alleles of a gene (e.g., *AA* or *aa*), and the term **heterozygous** is used to describe an animal that inherited differing alleles of a gene from each of its parents (e.g., *Aa*). Alleles can be **recessive**, meaning that an animal must inherit the same allele from both parents (i.e., be homozygous) before there is an effect, **additive** inheritance means that the effect on phenotype is proportional to the number of alleles that are inherited by the animal (i.e., inheriting two copies of a particular allele produces double the effect of inheriting one copy), or **dominant** meaning that the inheritance of a single dominant allele can completely mask the expression of the allele inherited from the other parent. Certain coat color phenotypes provide an example of complete dominance where, for example, black coat color (*B*) is completely dominant to red coat color (*b*). Crossing a homozygous dominant "*BB*" black bull to a homozygous recessive "*bb*" red cow will result in all heterozygous black "*Bb*" offspring. Most genetic defects are recessive, and the reason for this is that mutant alleles often render the resulting protein nonfunctional. These are called **loss of function alleles**.

It is important to understand that differences between individuals occur because of naturally-occurring mutations in the DNA sequence and that this variation provides the basis for selection programs and genetic improvement. Mutations are not always associated with decreased fitness; in fact mutations provide the starting variation for adaptive evolution. Mutations are relatively common, occurring every generation. Every mammalian progeny inherits a set of chromosomes from each of its parents that contain about 30 nucleotides that differ from the DNA sequence of each parent. It has been estimated that the average human carries approximately 250-300 loss of function mutations in known genes, and that 50-100 of these mutations have previously been implicated in inherited disorders. In many cases, if an individual inherits a functional allele from one parent, there is no phenotype associated with inheriting the loss of function mutant allele from the other parent. As such a heterozygous "*Aa*" (*A* symbolizes the dominant allele, *a* the recessive allele) animal, or carrier, appears normal. Because carriers appear normal, recessive alleles can increase in frequency in a population more easily than undesirable dominant or additive alleles. It is only when two carriers (*Aa* heterozygotes) mate that there is the possibility of producing offspring that have by chance inherited both of the non-functional alleles from their parents. The gene combinations that can occur with a recessive genetic condition are shown in Figure 1.



**Figure 1.** Mating combinations that are possible for an autosomal (non sex-associated) recessive genetic condition. Free means that the animal possesses two functional copies of the allele. Affected means two loss-of-function copies. Note that if this locus was a genetic lethal then all of the animals that are represented as solid black would die and so the only possible matings would be between free (green) and carrier (green and red) individuals.

The term **genotyping** refers to the process of using laboratory methods to determine which alleles an individual animal possesses, historically at one particular gene or “locus” in the genome. The **genotype** identifies which alleles each animal possesses. The DNA lab will need to receive a sample of tissue from which to extract DNA from the animal you wish to test. Because nearly all cells contain DNA, it is possible to genotype from many different tissue types. Laboratories may differ for their preferred sample type. Typically, samples include blood vials or cards, semen, or tail hair samples. It is important that tail hair samples include the roots – ideally 30-50 hairs with intact root bulbs from the switch of the tail. If you are sampling DNA from a deceased animal, call the testing laboratory to determine the best protocol. It is important to get a good quality sample to ensure that the DNA test will run successfully. The list of genetic conditions currently being monitored by U.S. beef breed associations is shown in Table 1.

<i>Genetic Abnormality</i>	<i>Primary Breed(s) of Incidence</i>	<i>Lethal or Nonlethal</i>
Alpha ( $\alpha$ )-Mannosidosis (MA)	Red Angus	Lethal
Arthrogyposis Multiplex (AM)	Angus	Lethal
Beta ( $\beta$ )-Mannosidosis	Salers	Lethal
Bovine Blood Coagulation Factor XIII Deficiency (F13)	Wagyu	Nonlethal
Chediak-Higashi Syndrome (CHS)	Wagyu	Nonlethal
Claudin 16 Deficiency (CL16)	Wagyu	Nonlethal
Contractural Arachnodactyly (CA)	Angus	Nonlethal
Developmental Duplication (DD)	Angus	Nonlethal(?)/ incomplete penetrance
Dwarfism (D2)	Angus	Nonlethal
Bulldog Dwarfism (BD)/ (Chondrodysplasia)	Dexter	Lethal
Erythrocyte Membrane Protein Band III Deficiency (Spherocytosis) (Band 3)	Wagyu	Often lethal
Hypotrichosis (hairless calf)	Hereford	Nonlethal
Factor XI Deficiency (F11)	Wagyu	Nonlethal
Freemartin (FM)	All	Sterile female <sup>2</sup>
Idiopathic Epilepsy (IE)	Hereford	Nonlethal
Neuropathic Hydrocephalus (NH)	Angus	Lethal
Osteopetrosis (OS)	Angus and Red Angus	Lethal
Protoporphyrria	Limousin	Nonlethal
Pulmonary Hypoplasia and Anasarca (PHA)	Maine-Anjou and Shorthorn; Dexter	Lethal
Tibial Hemimelia (TH)	Shorthorn and Maine-Anjou; Galloway	Lethal

The cost of testing varies depending upon the company and how many tests are performed but ranges from \$10-40/test; with an average of ~\$25/test. Irrespective of carrier animals in its pedigree, an animal that has been tested and found to be a non-carrier did not inherit the mutant allele and will not transmit the genetic defect to its progeny. It is for this reason that we should not prohibit the use of carrier animals within breeding programs. One half of the progeny of carrier animals will be free of the defect causing mutation.

<sup>2</sup> Technically this is not a recessive condition but rather infertility resulting from sharing the womb with a male twin.

In fact, all animals are carriers of mutations somewhere in their DNA which cause recessive defects. Because an animal must inherit two copies of a given recessive mutation to be affected, and if only a few animals in the whole population typically carry the same mutation, there is rarely a mating that has the potential to create an affected offspring. It is when the frequency of recessive mutation is high as typically happens when relatives are mated that there is a greater possibility that offspring will inherit the mutant allele from both sides of the family tree.

### **Inbreeding**

Inbreeding is the mating of animals that are more closely related to each other than the average degree of relationship in a population. Inbreeding tends to increase the extent of genetic homozygosity. Its main purpose is to make inbred animals more homozygous for superior genes, however deleterious allelic variants also become homozygous at the same time unless such variants are homozygous lethal in which case there is obvious natural selection against them. Inbreeding results from one or more ancestors having contributed identical alleles on both the male and female sides of an animal's pedigree. It is for this reason that deleterious autosomal recessive variants are often identified in the descendants of widely used sire lines. It is not because sires with excellent genetic merit carry more deleterious variants, it is because such sires are more likely to be represented on both sides of a pedigree opening up the possibility that an animal will inherit a deleterious allele from both its sire and dam. In populations that have been inbred over a period of time, animals can become so related in general that matings between distant relatives may in fact be matings of closely related individuals at the level of their DNA.

The **inbreeding coefficient** measures the average extent of additional chromosomal homozygosity, relative to the average of the population from which the animal came. In an inbred animal this is due to the relationship between its parents. Some inbreeding rates, expressed as percentages, are shown in Table 2. Inbreeding promotes an increase in prepotency, which the ability of an individual to produce progeny whose performance is especially like its own and/or is especially uniform. This results from the increase in homozygosity. Because inbred individuals have fewer heterozygous loci they cannot produce as many genetically different sperm or eggs. The result is less variation in the offspring.

<b>Mating</b>	<b>Inbreeding coefficient of offspring (%)</b>
Sire x Daughter	25.0
Sire x Sire's dam	25.0
Full-sibs	25.0
Half-sibs	12.5
Sire x Granddaughter	12.5
Son of a sire x Granddaughter of the sire	6.25
Grandson of a sire x Granddaughter of the sire	3.13

However, inbreeding is also accompanied by **inbreeding depression** - the decline in average phenotypic performance that occurs due to the inbreeding. This phenomenon is well documented and has the greatest effect on reproductive traits, followed by growth traits. In beef cattle, inbreeding depression has been documented for many traits including percentage weaned, birth weight, preweaning gain, weaning weight, postweaning gain, and final weight. When the effect of inbreeding was assessed as a percentage of the mean, it was more pronounced for lifetime number of calvings, weight at three months, and productive longevity, intermediate for other reproductive traits and weights of calves, but was not significant for feed to gain ratio, or carcass composition. In general life-history and fitness-related traits are those where inbreeding depression has a larger effect.

### **Management Options for autosomal recessive conditions**

Before genetic testing, the only way to identify carriers of autosomal recessive genetic conditions was to perform a test cross with other known carriers and observe whether any of the offspring were homozygous affected. Perhaps the most famous example of a genetic defect in 20<sup>th</sup> century beef breeding was “snorter” dwarfism which became an issue in Angus and Hereford cattle during the 1940s and 1950s. This genetic defect was uncovered as a result of strong selection pressure for animals with small stature. Ultimately, the cause of this mutation was traced back to a bull named St. Louis Lad, born in 1899. A 1956 survey of Hereford breeders in the USA identified 50,000 dwarf-producing animals in 47 states. Through detailed pedigree analysis and test crosses, the American Hereford Association, in concert with breeders and scientists, virtually eliminated the problem from the breed. Because carrier status was difficult to prove and required expensive progeny testing, some entire breeding lines were eliminated which contributed to a loss of diversity and an increase in overall inbreeding within the breed. This situation can be contrasted to the speed with which genetic testing has allowed 21<sup>st</sup> century breeders to quickly and accurately determine the carrier-status of their animals and manage their use within breeding programs.

There are many management options for the control of genetic conditions and the best choice will depend on the needs and requirements of each enterprise. Some of these options include:

1. Testing all animals and culling carriers.
2. Testing all animals and using carriers only in terminal breeding programs or in matings to non-carrier animals and testing their progeny.
3. Testing sires and using only free bulls for breeding.

This last option will eliminate affected progeny and decrease the number of carriers over time. However in some cases, the overall breeding value of carrier animals may outweigh the economic penalty associated with their carrier status. Carrier sires and dams can be used for breeding. However, follow-up testing of all progeny is essential to develop future breeding strategies with these progeny, because we would expect 50% of their progeny to themselves be carriers. These carriers can be identified by DNA testing. They may be culled, or used in a separate breeding programme with animals of identified negative or free status. The negative status progeny can then be used to perpetuate phenotypically and/or quantitatively selected superior production traits. Continued breeding of two carriers risks the production of approximately 25% affected animals.

Crossbreeding offers another management approach to address deleterious alleles. First crosses are unlikely to produce any affected progeny (unless both breeds have the same deleterious allele), but there is a risk of increasing the prevalence of the defective gene in the carrier state. Subsequent interbreeding of cross-bred animals has the potential to produce additional carriers and affected cases.

The Angus breed has recently had to manage several simply-inherited, single locus recessive genetic conditions. These include two lethal conditions Arthrogyrosis Multiplex (AM; “Curly Calf Syndrome”), and Neuropathic Hydrocephalus (NH). AM is caused by a chromosomal deletion that occurred in Rito 9J9 of B156 7T26, (AA Registration No. 9682589; born 29 October 1979). NH occurred as a result of a single DNA base pair mutation in his grandson, the widely-used GAR Precision 1680 (AA Registration No. 11520398; born 6 September 1990). The widespread use of this bull spurred on by an increased selection emphasis on carcass traits increased the probability of this bull showing up on both sides of many Angus pedigrees, thereby uncovering the presence of recessive lethal mutations throughout the genome of this bull. The third condition is a non-lethal autosomal genetic defect called Congenital Contractural Arachnodactyly (CA; “Fawn Calf Syndrome”) that is

caused by a deletion of around 54 kbp. An additional condition called developmental duplication (DD) was identified in 2013. This condition has incomplete penetrance, meaning that there are some animals that are homozygous for the mutation that causes DD that do not have the DD phenotype. Incomplete penetrance is a fairly new phenomenon to beef cattle breeders but is well known and fairly common in human genetic diseases. It occurs because the disease is not really caused by a single gene defect. In fact, the disease is caused by interactions among several genes and if you have the “right” combination of alleles at other loci you are protected from expressing the disease phenotype when you inherit two disease causing alleles at the major risk locus. Other breeds have their own suite of genetic conditions, as would be expected from the knowledge that all individuals carry deleterious mutations. The identification of such mutations increases when certain sires are used extensively such that deleterious alleles come together through inbreeding on both sides of an animal’s pedigree.

Genetic tests are available for many genetic conditions in different breeds (Table 1). The speed with which these genetic tests were developed is a testament to the power of having access to the bovine genome sequence information, and is perhaps the greatest success story of genomics never told. The proactive response of the breed association in making genotypes available also helped to rapidly and transparently address the problem. It is important to realize that although genetic defects can be problematic for individual breeders, they do not have to be catastrophic for the industry. The sooner a defect is recognized and the genetic cause identified, the sooner breeders can use this information to avoid producing affected individuals and eventually reduce or eradicate the allele from the population. The genetic status of a registered animal can be determined by looking at the breed association website.

Different breeds report their results in a variety of formats but typically there will be information stating whether a tested animal is a carrier, an affected homozygote, or a free homozygote. For example, on the American Angus website the designations are as follows:

- P - Refers to a "potential" carrier based on an ancestor known to carry that specific mutation.
- F - Refers to an animal tested for one or more genetic conditions and determined to be "free" of that specific mutation; or one that has never had any carrier animals in its pedigree.
- C - Refers to an animal tested for one or more genetic conditions and determined to be a “carrier” of that specific mutation.
- A - Refers to an animal tested for one or more genetic conditions and determined to be a carrier of two copies of that specific mutation (i.e. affected). It may or may not exhibit the phenotype associated with that genetic condition, depending on whether the condition is fully penetrant.

A free “F” homozygote will not pass on the genetic defect, irrespective of the prevalence of that defect in their pedigree. Carriers will pass on the defective allele to half of their progeny. It is possible to estimate the likelihood that a potential carrier inherited a defective allele, but testing is the only way to confirm the true genotype of an animal for that locus.

### **The future**

There are several groups throughout the world working on trying to identify the actual SNP or quantitative trait nucleotide (QTN) genetic cause of lethal recessive conditions. This is being done through sequencing of the 3 billion base pair genomes of prominent AI bulls in many different breeds. The research team at University of Missouri led by Dr. Jerry Taylor is sequencing bulls from the following breeds of cattle (Table 3).

<b>Breed</b>	<b>N</b>	<b>Number of Reads</b>	<b>Total Bases</b>	<b>Total Coverage</b>	<b>Avg Coverage</b>
Angus	99	77,930,820,090	7,694,958,893,355	<b>2,653</b>	27
Red Angus	14	4,430,950,144	441,846,880,499	152	11
Hereford	18	14,775,544,682	1,390,024,023,122	479	27
Limousin	12	3,704,169,818	357,264,463,240	123	10
Charolais	11	8,061,833,430	802,164,255,493	277	25
Simmental	11	8,902,705,282	885,698,817,042	305	28
Gelbvieh	8	6,366,906,096	633,479,558,830	218	27
Maine Anjou	5	4,061,220,172	403,867,224,031	139	28
Romagnola	4	901,554,762	89,666,842,589	31	8
Shorthorn	2	1,370,128,728	136,274,291,678	47	24
Beefmaster	10	8,351,392,646	830,865,082,100	287	29
Holstein	25	3,224,948,436	320,796,806,908	111	4
Jersey	9	1,399,450,902	139,150,036,295	48	5
N'Dama	1	739,233,320	73,483,493,461	25	25
Brahman	11	1,871,667,422	167,772,161,118	58	5
Nelore	8	1,668,006,036	165,728,918,125	57	7
Gir	6	1,583,737,248	157,449,065,756	54	9
Bison	3	3,242,100,744	322,544,004,793	111	3
<b>Total</b>	<b>257</b>	<b>152,586,369,958</b>	<b>15,013,034,818,435</b>		

It can be seen that there are enormous amounts of data involved in these sequencing projects. The research team is looking for mutations that would be predicted to either damage or totally eliminate the function of a gene. They will then cross-compare these with essential gene (certain genes that are known to be required for life) databases from human and mouse and see if there is any overlap. Mutations in these essential genes are most likely to be those that underlie fertility in beef cattle.

Using these data, a research DNA test will be developed to enable the genotyping of a large population of heifers. Dr. Dave Patterson is coordinating the DNA collection of these 10,000 heifers as part of the Show Me Select heifer development program (<http://agebb.missouri.edu/select>). These animals will be genotyped and the prevalence of the damaged/mutated alleles will be tallied. Some genes will be apparently non-functional, but we may still observe live, homozygous animals because these genes are not essential for life. On the other hand, they are likely to reduce the growth performance of these animals. On the other hand, if homozygotes are completely missing for any of the identified mutations, this will be very strong evidence that the loss of function allele is lethal and all homozygous embryos abort. The proposed outcome of this grant will be a chip that includes all of the recessive alleles that result in embryonic lethality, and animals carrying these alleles will be candidates for more careful management to avoid fertility reductions due to early embryonic loss.

These research efforts are likely to identify a large number of deleterious or lethal recessive alleles in beef cattle populations. Although such alleles may occur at low frequencies, their combined effect on fertility may be important and consideration of these known genetic conditions should be included in selection and breeding programs in the future. Decision support software will be essential to manage all of this information by providing an objective approach for appropriately weighting the relative economic value of traits in the breeding objective against potential deleterious recessives, and suggest an optimal breeding scheme based on all of the available information.

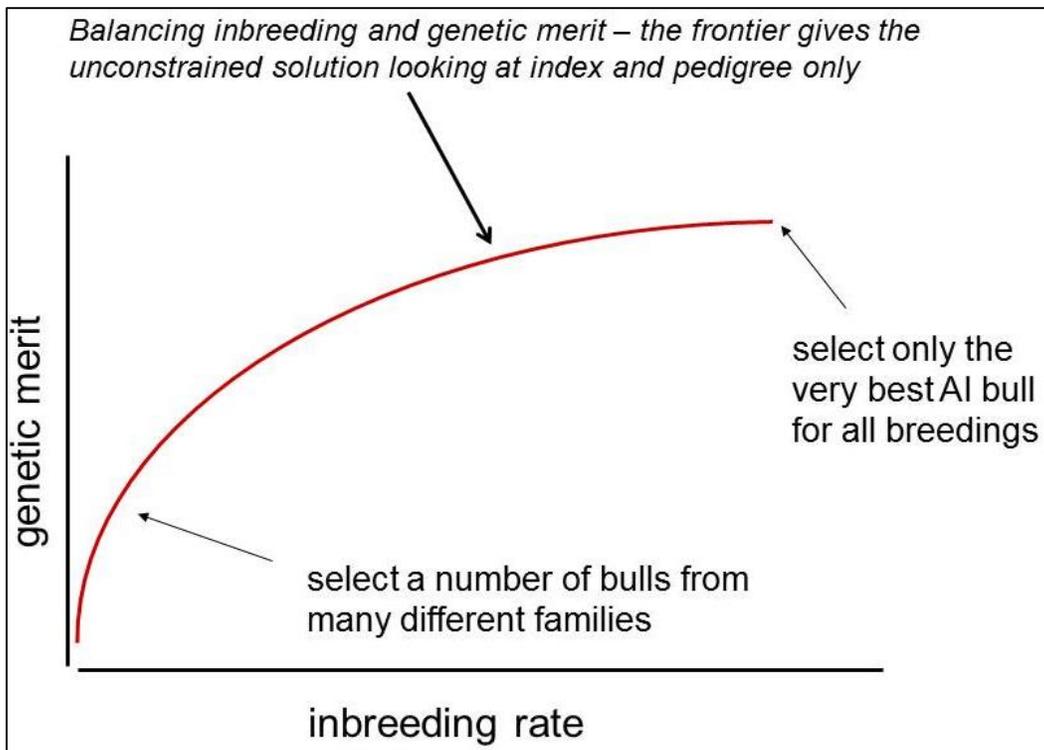
## Management of lethal recessive conditions

Management of lethal recessive conditions that result in embryonic mortality requires special consideration. This case is important because unlike a calf born with an obvious physical deformity, these conditions manifest as fertility problems presenting as an increased calving interval or missed heat(s). From a genetic perspective there will be a statistically significant absence of living progeny homozygous for the causal allele. In several dairy cattle breeds, the absence of homozygous genetic markers amongst thousands of genotyped progeny has been used to identify several lethal autosomal recessive alleles that impact fertility.

Lethal alleles can be optimally managed through systems of mating. These constitute breeding plans that are designed to combine the genes in a population in the most advantageous genotypic combinations. For example mating, a known carrier in one breed to cows from another breed that do not possess the allele will not result in any affected progeny, allowing the genetic merit of that carrier to be utilized without resulting in affected progeny. Likewise, carriers of two different recessive alleles at different loci can be crossed and will not produce any affected offspring for either condition. This concept of mate selection to optimize genetic progress while focusing on reducing the incidence of affected individuals is likely to become increasingly important as genomics identifies an ever-increasing number of deleterious recessive alleles in domestic livestock populations.

Management of recessive conditions has to be balanced with other important issues such as the management of trait merit, genetic diversity, other genetic defects, genome-wide inbreeding, logistical constraints and costs. It is likely that decision support software will be required to facilitate the management of this information. The dairy industry has already developed some mate allocation and decision support software that allows breeders the opportunity to selectively use recessive lethal carrier sires that also transmit breed-leading profitable traits with cows that are free of the deleterious allele. These programs provide an approach to make judicious use of carrier bulls with superior genetic merit while avoiding the production of affected calves, and while strategically working to slowly eradicate the allele from the population. There is a need to utilize carrier animals appropriately rather than prohibit their use entirely and let mate selection software optimize their use in the breeding programs.

Dr. Brian Kinghorn at the University of New England in Australia, has developed a software program called MateSel which makes the optimum mate selections (i.e., maximize genetic gain while minimizing the loss of genetic diversity). Figure 2 shows the tradeoff between genetic gain achieved by mating the very best sire to the very best dams irrespective of their relationship to each other, and genetic diversity which is maintained by breeding a large number of bulls from different families (i.e., decrease inbreeding rate). As a part of the aforementioned USDA grant, Dr. Kinghorn is in the process of adapting mate selection methodology and modifying MateSel so that it can be used to optimize the rate of genetic gain and mate allocation with a key objective being to reduce both affected offspring (i.e., “*aa*” calves) and allele frequency of the lethal alleles identified in the project. This will be done by including two additional parameters in the mate selection software; 1) the predicted number of recessive lethal alleles, across nominated loci, in the progeny of each mating (**LethalA**); and the predicted number of recessive lethal genotypes (i.e., *aa*) in the progeny of each mating (**LethalG**) across all loci.



**Figure 2.** The tradeoff between accelerating the rate of genetic gain and inbreeding rate.

Using this software, LethalA can be used to select against the number of recessive lethal alleles in the population, i.e., decrease the number of carriers. LethalG can be used to select against the incidence of lethally affected progeny from the current matings. It is really this latter number that results in loss because the embryo does not survive. Carrier embryos do not incur a loss and simulations have shown that using a selection strategy that focuses only on avoiding “aa” offspring (i.e., LethalG) achieved a superior outcome in terms of decreased impact on rate of genetic gain and reduction in the numbers of progeny lost as compared to selecting against the presence of carrier offspring (i.e., LethalA). In other words, using mate selection software to avoid carrier matings achieves a better outcome than just indiscriminately avoiding the use of carriers.

### **Summary and Implications**

Ultimately as gene discovery continues, it is likely that we will find that all animals are carriers of alleles which cause undesirable phenotypes, even if this is reduced growth potential. Recessive loss of function lethal alleles should always be expected, but they are usually at low frequency and can be managed to reduce the incidence of affected offspring. Identifying carrier animals enables breeders to avoid having embryonic losses by tactical mate selection. The bottom line is that every animal carries genetic defects and typically breeders do not know what they are or where they are located in the genome. The objective of this research project is to identify some of these alleles and to develop tools for the implementation of strategic mating. This will help ensure that the value of high genetic merit carriers be maintained as tools will be available to appropriately utilize those genetics in breeding programs. Ultimately, the goal of this project is to enable more cows and heifers to get settled on their first breeding--which has many positive resultant knock-on benefits. Given the value of fertility to cattle operations, even a small increase in conception rates on the first breeding would have considerable value to the beef cattle industry.

# Opportunities for Embryo Genotyping and the Impact on Pregnancy Creation

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Embryo genotyping, by way of biopsy procedure, is a viable and accurate diagnostic technique to acquire genetic information at the embryo level. Currently, this method of attaining genetic data in beef cattle has been limited to testing for and diagnosing certain traits and characteristics. By definition, it is used to identify genetic defect status (carrier versus affected embryos) and embryo gender determination. This laboratory procedure results in isolating the genetic status of >90% of the embryos tested. Since the DNA analysis requires several days to complete, embryo freezing post-biopsy is the most common practice followed. Those embryos which are biopsied for DNA analysis, frozen, and subsequently thawed, create pregnancies at a rate of 7-10% lower than non-biopsied frozen/thawed embryos.

## INTRODUCTION

Renowned pathologist, Dr. Horst Leipold, acknowledge that by the 1980's there had been 158 identifiable congenital (present at birth) defects in cattle (Leipold and Dennis, 1980). Of these known defects, over 50 had been proven to be heritable traits and therefore, were being passed to offspring genetically. However, these defects only have become apparent as animals that are genetic defect carriers for a particular trait are mated to other genetic defect carriers of the same trait. A portion of the resulting calves are destined to display the physical defects and the beef industry has been challenged with the identification of several of these genetic flaws. As a way to combat the devastating results of genetic defects, the beef industry relied on embryo transfer and embryo genotyping (DNA analysis) to accurately identify the presence of DNA alterations. Employing this type of technology resulted in providing a way to quickly identify those embryos that would be carriers of the genetic aberrations (or in some cases be affected by the genetic defect).

As genetic defects were unearthed, many avenues were tested to obtain further information to identify the source of the genetic faults. Initial investigations in the 20<sup>th</sup> century relied on astute observation, detailed record-keeping, and communication amongst investigators. A great deal of time was spent ruling out environmental causes of congenital defects such as viruses or toxins. As time progressed, investigators found that progeny testing was an efficient way to identify genetic problems (Leipold and Dennis, 1980). Consequently, Dr. Leipold maintained a large herd of beef cattle that phenotypically displayed a concerning genetic defect (syndactyly; more commonly known as "mule foot" in which the two toes are fused to create one that resembles that of a mule or horse). Mating cows, known to possess this physical defect with suspect semen, resulted in a way to progeny test bulls for the respective genetic defect. This ability to directly progeny test sires reduced the time interval of genetic determination from years to one genetic interval thus leading to quicker genetic improvement.

Rapid genetic detection didn't stop at live progeny testing using test bulls to breed genetically defective cows. Dr. Leipold again utilized reproductive techniques to progeny test carriers via embryo transfer. By producing multiple embryos in test matings and harvesting 60-day old fetuses via c-section, Dr. Leipold and colleagues were able to reduce the time interval of genetic determination from a generation to months (Johnson et. al., 1985). Today, after much advancement, embryo biopsy reduces this time interval of genetic determination from months to merely days. Embryo genotyping has taken this analysis to the next level by predicting which embryos have genetic defects present. This allows cattle producers to envisage and prevent the birth of genetically defective/affected calves.

## PROCEDURE

The process of embryo biopsy begins with identifying the carrier dams. Having the knowledge that a particular dam is defective in part of her genetic make-up allows cattle breeders to select complimentary sires to aid in limiting the number of affected embryos. Embryos are collected on day 7 (day 0: breeding via artificial insemination or natural service). For biopsy, it is optimal to work with embryos collected on day 7 as they demonstrate the proper morphology to be biopsied. These embryos possess a compact inner cell mass and are not advanced beyond a stage 5 of development. These ideal embryos are identified and transferred to a

working plate separate from embryos collected from other donors within the same group. It is imperative that the biopsy procedure is conducted in a controlled environment, away from environmental effects such as laboratory traffic and equipment interference, to maintain the healthy integrity of the embryos.

All embryos are rinsed, staged and graded, and further cleaned off with a media<sup>1</sup> unique to the biopsy process to make the biopsy action possible. As embryos are spherical in nature, they tend to roll around in their wells when greeted with the splitting blade. This specific media aids in keeping the embryos “attached” to the bottom of the plate so they may properly be biopsied. Once prepared, an embryo is loaded into a single media droplet on a working dish and transferred to the micro-manipulator<sup>2</sup>. The blade is lowered to just above the embryo using a motorized joystick and proper placement is verified. When biopsying embryos only 6-10 cells are required to be removed for DNA analysis. Further lowering of the blade will not only cut through the zona pellucida but will also split the cell mass into two parts; proper placement will result in 6-10 cells being separated from the remainder. The blade is raised and the working plate is returned to a dissecting scope. Before biopsying the next embryo, it is of utmost importance the blade is rinsed with alcohol, deionized water, and then splitting media to prevent DNA contamination between embryos. Here the biopsied piece is located and transferred into a 96-well hard-shell PCR plate with 5 microliters of holding media<sup>3</sup>. In the working dish, the remaining embryo mass is located and placed into a different loading plate and waits for freezing preparation while the remaining embryos are biopsied following the same steps previously mentioned. Once all the embryos are biopsied and the respective pieces are loaded properly into a PCR plate, the PCR plate is sealed and frozen in a refrigerator/freezer prior to shipping. All PCR plates are shipped to Dr. Jonathan Beever for analysis at the University of Illinois<sup>4</sup>. The remaining embryos are loaded into ¼ cc direct transfer straws with individual identifying labels attached with an impact heat sealer. The embryos are frozen in the similar manner of conventional whole embryos in ethylene glycol<sup>5</sup> (seeded at -6°Celsius and cooled to -35°Celsius at 0.5°Celsius per minute). Following this cooling process, embryo straws are plunged into liquid nitrogen and stored in canes in nitrogen tanks, indefinitely.

## RESULTS & DISCUSSION

The use of embryo biopsy has been used in our lab to identify embryo status (carrier/affected) of embryos for six genetic defect traits and for gender determination (table 1.0). The goals of this program were as follows:

- Biopsy all embryos from a suspect collection
- Accomplish at least 90% genetic status determination with DNA testing
- Maintain an acceptable pregnancy rate with the biopsy/frozen/thawed embryos

Historical research has demonstrated that 92 - 94% of genetic data of those embryos tested is attainable (Lopes et. al., 2001). In table 1.0 below are the results of genetic defect testing accomplished by our clinic in cooperation with Dr. Beever since 2009 and the resulting DNA analysis results; while table 2.0 indicates genetic testing for gender determination that was completed in conjunction with the defect testing.

<b>Defect</b>	<b>No. Collections</b>	<b>Total No. Analyzed</b>	<b>No. Free</b>	<b>No. Carrier</b>	<b>No. Non-Determined</b>
<b>Arthrogryposis Multiplex (AM)</b>	92	740	385	292	63
<b>Neuropathic Hydrocephalus (NH)</b>	92	669	322	291	56
<b>Idiopathic Epilepsy (IE)</b>	8	56	30	23	3
<b>Developmental Duplication (DD)</b>	2	11	5	5	1
<b>Contractural Arachnodactyly (CA)</b>	2	9	3	6	0
<b>Tibia hemimelia/Pulmonary hypoplasia with anasarca (TH/PHA)</b>	2	14	1*	11**	2
<b>Total</b>	198	1499	745	617	125 (8.33%)

\* Free of both TH and PHA, \*\* Carrier of TH or PHA, or both, or affected with TH or PHA

<sup>1</sup>Bioniche Vigro™ Splitting Plus, EVM062

<sup>2</sup>Bioniche Twinner System Bionocular, ESE010

<sup>3</sup>Bioniche Vigro™ Holding Plus, EVM024

<sup>4</sup>Beever, Jonathon PhD, Marron, Brandy MS, Laboratory of Molecular Genetics, University of Illinois. 1201 West Gregory Drive, RM 220 ERML, Urbana, Ill 61801.

<sup>5</sup>Bioniche Vigro™ Ethylene Glycol Plus, with sucrose, EVM034

No. Collections	Total No. Analyzed	No. Males	No. Females	No. Non-determined
202	1562	748	678	136 (8.71%)

Embryo biopsies were sent to Dr. Beever's lab, requesting 3061 DNA tests (Genetic defects, n=1,499; Gender determination, n=1,562). Of those sent for analysis, 261 did not yield any results (defect analysis or gender determination). Embryo samples (n= 2800) did produce genetic results (91.5%) thus meeting our goal of at least 90% attainable results. These data indicated the embryo biopsy technique was adequate for supplying DNA material for the PCR analysis performed by Dr. Beever's lab.

The genetic defects tested for, specifically AM and NH, are considered simple autosomal recessive traits. The AM and NH testing yielded 57.0%, 52.5% free and 43.0%, 47.5% carrier embryos, respectively. The deviation in both tests from the expected 50/50 ratio does not appear to be significant and will not be discussed here. The IE, DD, and CA test values were nominal; evaluation of the ratios is unwarranted. In the case of the TH/PHA tests, small values did not allow for any ratio evaluation; however, testing for two defects gives several possibly outcomes. Included in the TH/PHA results are actual "affected" results, meaning those defect (affected) embryos would be born dead, with the physical defect. Gender determination rate was 91.3%, with 52.5% being males and 47.5% being females. This gender ratio appears to be similar to the data reported previously (Shea, 1999).

Pregnancy data for those embryos which were biopsied, frozen, thawed, and then transferred are shown below (table 3.0). Overall pregnancy results from these embryos created pregnancies at a 48.18% rate. This rate represents more embryo loss than desired. Few embryos were transferred in our program, with the bulk of the embryos being shipped to the respective owners; therefore, further review of additional pregnancy creation data has been difficult to accomplish. Pregnancy creation in our program proved the procedure is damaging to the embryo, specifically with post-biopsy freezing. Small numbers of embryos were transferred immediately after the biopsy (no freezing). The pregnancy rates appeared to not be affected by the surgical procedure as numbers are similar to those previously reported (Shea, 1999; Lopes et. al., 2001). Also, some embryos were thawed, then biopsied prior to transfer. Again, our pregnancy rates appeared to not be affected by the surgical procedure when compared to previous data (Shea, 1999; Lopes et. al., 2001). Using the biopsy technique in this manner may have pregnancy creation merit; however, pregnancies will be created that have the defective DNA. This results in short-term pregnancy termination, which is a significant cost factor in our program and our clients' programs.

Defect Free Category	No. Transfers	No. Pregnant	No. Open
AM	193	95	98
NH	62	30	32
Gender only	19	7	12
<b>Total</b>	274	132 (48.2%)	142 (51.8%)

Embryo biopsy has proven to be a viable technique to supply DNA material for genotyping. Genetic loss associated with reduced pregnancy creation does occur as a result of this surgical procedure. However, the continued use of embryo biopsy for genotyping is dependent on other factors unrelated to the results of the procedure. The genetic defects that surfaced in 2008-2009 (AM and NH) were lethal defects. Calves affected with these defects were either spontaneously aborted during gestation or were still born. This pregnancy/calf loss is very costly to the beef industry. Breed associations ruled at that time, any calf born to an AM or NH carrier mating must be tested for the genetic defect, and calves carrying the defect could not be registered (<http://www.angus.org/Pub/GeneticConditions.aspx>). This ruling forced breeders to avoid creating defect-carrier calves.

## **SUMMARY**

Embryo biopsy and DNA analysis has given breeders an avenue to continue producing calves by identifying and avoiding the use of embryos that were proven to be carriers of the genetic defect. Breed association rulings for non-lethal defects, such as CA or DD, have been less restrictive to the breeders. Cattle breeders feel less pressure to subject their program to this embryo identification process, as is evidenced by the number of collections for biopsy with CA (n=2) and DD (n=2).

Continued cattle breeder use of embryo biopsy for genotyping, specifically for genetic defect identification will depend on the following factors: 1) Genetic defect identification that is lethal, 2) Breed association rulings that restrict calf registration, and 3) Genetic value within a herd and/or the market place. Embryo biopsy for gender determination will continue to be used as particular matings are designed to only create calves of a specific gender for a specific market. The use of embryo biopsy will compete with the continued increase in the use of gender specific semen available to the cattle breeding industry today.

## **References**

Johnson, J. L., Leipold, H.W., and Hudson, D.B. 1985. G85-759 Prominent Congenital Defects in Nebraska Beef Cattle. *Historical Materials from University of Nebraska-Lincoln Extension*. Paper 319.

Leipold, H.W., Dennis, S.M. 1980. Congenital Defects Affecting Bovine Reproduction. Pages 410-441 in *Current Therapy in Theriogenology*.

Lopes, R.F., Forell, F., Oliveira, A.T., Rodrigues, J.L. 2001. Splitting and biopsy for bovine embryo sexing under field conditions. *Theriogenology* 56(9); 1383-92.

Shea, B.F. 1999. Determining the Sex of Bovine Embryos using Polymerase Chain Reaction Results: A Six-Year Retrospective Study. *Theriogenology* 51(4):841-854.