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## Ancestral Developmental Potential: a new tool for animal breeding?

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*“... I wish to guard the reader against supposing that reversion is due to some rare or accidental combination of circumstances. When a character, lost during hundreds of generations, suddenly reappears, no doubt some combination must occur; but reversions may constantly be observed, at least to the immediately preceding generations, in the offspring of most unions ... Reversion is most likely the rule, as Mr Sedgwick has shown, with certain diseases...”*

This insightful quote from Darwin’s (1868) classic *The Variation of Animals and Plants under Domestication* illustrates that although a trait is lost during the evolution of a lineage, the potential to produce that trait is retained, such that it may reappear in individuals in modern populations. Whales and dolphins, for example, are closely related to hoofed animals but lost their hind limbs ~34-41 million years ago when they re-entered water

(Thewissen et al., 2006). Today, several anomalous individuals of this group have been discovered with partial hind limbs indicating that hind limbs have “reappeared” in these individuals (Hall, 2003; Tomić & Meyer-Rochow, 2011). The sudden reappearance of such ancestral traits in these anomalous individuals is most often called “atavism,” which is derived from the word “atavus” or ancestor (Darwin 1868). Atavistic traits can suddenly reappear in individuals even though they had been lost for hundreds, thousands, and even millions of years (Collin & Miglietta, 2008; Wiens, 2011).

Unfortunately, modern evolutionary biologists appear to have undervalued the true significance of atavisms for evolutionary theory. The consensus view is that atavisms are rare mistakes in the developmental system that only provides evidence of ancestry (Levinton, 1986). Apparently, as stated above in his quote, Darwin failed in “*guarding the reader against supposing that reversion is due to some rare or accidental combination of circumstances*” and as a consequence, atavisms are currently viewed as rare “freaks of nature” that contribute little to the raw material that natural selection can act upon (Blumberg, 2010). We argue in the following paragraphs that Darwin was right about his observation that reversions/atavisms are a common occurrence in nature and that this type of variation is likely to be “the rule” and not the exception. This is especially true for poultry, where ancestral traits frequently reappear in individuals in modern populations and can also be induced experimentally (Darwin 1868; Harris et al., 2006; Hampe, 1959; Müller, 1989). Furthermore, we summarize evidence from our recent study (Rajakumar et al. 2012) showing that reversions/atavisms reflect “ancestral developmental potentials” that, when induced, provide raw material that natural selection can act upon to facilitate adaptive evolution. Giving reversions/atavisms their proper place in evolutionary theory (West-Eberhard, 2003; Stiasny, 2003), brings forth entirely new perspectives on how “ancestral developmental potentials” can be used to improve animal breeding and understand complex disease, especially with respect to poultry. We will finish by briefly outlining these perspectives.

**Reversions/Atavisms occur frequently in poultry.** The field of experimental embryology provided some of the first evidence that atavisms in poultry can be experimentally induced. First, Hampe (1959) and Müller (1989) inserted a physical barrier within a specific region of the chicken hindlimb during its development. The outcome of this experiment was startling

– the adult bone and musculature now resembled that of reptiles. Because birds descended from reptiles (Lee, 2001; Hugall et al., 2007), this classic experiment demonstrates that it is possible to revert the hindlimb in chickens to its reptilian state. Another celebrated example of atavism in poultry is the induction of teeth in *talpid2* mutant chickens. Although birds lost teeth approximately 70-80 million years ago, Harris et al. (2006) discovered that teeth had reappeared (or at least had initiated development) in *talpid2* mutant chickens. In both these examples, the atavistic traits had been lost for millions of years, showing that ancestral developmental potentials can be retained for vast periods of time. Darwin (1868), however, shows how these potentials can be frequently induced by natural means in populations after the trait has been lost for generations. Darwin (1868) states: *“The best yet simplest characters lying dormant are, perhaps, those previously given, in which chickens and young pigeons, raised from a cross between differentially colored birds, are at first of one color, but in a year or two acquire feathers of the color of the other parent; for in this case the tendency to a change in plumage is clearly latent in the young bird.”* These examples of atavism illustrate that ancestral developmental potentials are retained for variable lengths of time and in many traits. Indeed, we have just scratched the surface in terms of surveying the ancestral potentials that exist in poultry.

**“Supersoldier ants” show that reversions/atavisms reflect ancestral developmental potential that can facilitate adaptive evolution.** Most new perspectives in science come from unexpected sources and in roundabout ways. In our case, we provide a new perspective on the biological significance of atavistic traits by studying the evolutionary developmental biology of ants. One of the most exciting discoveries to emerge from the field of evolutionary developmental biology (also known as EvoDevo) is the deep conservation, over hundreds of millions of years of evolution, of the genes that regulate development of an organism (Carroll, 2005; Carroll et al., 2009). For example, in all animals, including, ants, chickens, and humans, the developmental regulatory gene *hedgehog* is essential in the formation of limbs (Riddle et al., 1993; Ingham & McMahon, 2001), and the gene *Pax6* functions to specify where an eye will develop (Gehring & Ikeo, 1999). A team led by Walter Gehring conducted an experiment that beautifully illustrates the functional conservation of these developmental regulatory genes: they genetically inserted and expressed the mouse *Pax 6* gene in the developing wing or leg of a fruit fly, and

demonstrated that adult compound eyes (resembling those of a fruit fly) appear on wings or legs in the adult fly (Halder et al., 1995). How is it that these developmental regulatory genes, which are so conserved in their function and expression, can account for the amazing morphological diversity in the animal kingdom? The Abouheif Lab's approach to answering this fundamental question has been to study how these genes interact with their environment in the context of the complex societies of ants.

In the most advanced ant societies, the non-reproductive worker caste is very large, and can be composed of thousands and even millions of individuals (Hölldobler & Wilson, 1990; Wilson, 2003; Hölldobler & Wilson, 2009). The individual workers in these highly advanced societies are morphologically differentiated into several subcastes such that they can efficiently divide labor in the colony (Hölldobler & Wilson, 1990). In the genus *Pheidole*, which is one of the most evolutionarily diverse genera of ants with over 1000 species, the worker caste is divided into "minor workers" and "soldiers" (Wilson, 2003; Moreau, 2008). Minor workers forage and nurse the young, whereas soldiers defend the nest and help process food. In 8 of the ~1000 species in this genus a third "supersoldier" subcaste has evolved (Moreau, 2008). This supersoldier subcaste has a massive head which functions to block the nest entrance when attacked by army ants (Huang, 2010).

In our recent study (Rajakumar et al. 2012), we found that the ancestral species of all *Pheidole* had a supersoldier subcaste that was subsequently lost in most of the ~1000 species for ~30 to 65 million years. Supersoldiers then re-evolved multiple times independently in the genus. By applying a key growth hormone, called Juvenile Hormone, to larvae at a very specific time in development, we discovered that supersoldiers can be experimentally induced in species that had lost the supersoldier subcaste for over 30 to 65 million years (see fig. 3 & fig. S9 in Rajakumar et al. 2012). This means that although the supersoldier phenotype had been lost for millions of years, all *Pheidole* species retain an ancestral developmental potential to produce supersoldiers. We demonstrated that experimentally induced supersoldiers are produced through the same developmental pathway as naturally evolved supersoldiers, and as adults, both experimentally induced and naturally evolved supersoldiers are significantly larger than regular soldiers. The only distinct morphological difference we observed between experimentally induced and naturally evolved supersoldiers was the appearance of tiny wing vestiges on the thorax of those that were experimentally induced (See Fig. 3 in Rajakumar et al 2012). These wing

vestigial wings are thought to be detrimental; they do not allow individuals to efficiently maneuver underground, and therefore, are a negative side consequence of experimentally inducing supersoldiers. These tiny wing vestigial wings are important for understanding how ancestral developmental potential relates to complex disease, as we will soon describe below.

We also discovered in wild colonies of one *Pheidole* species, which does not have a supersoldier subcaste, several anomalous individuals that looked very similar to supersoldiers but also had tiny wing vestigial wings on the thorax (see fig. 2 Rajakumar et al. 2012). Furthermore, anomalous supersoldier-like individuals were also found in wild colonies by other researchers in different species (Wheeler, 1902; Goetsche, 1937), meaning that this ancestral developmental potential is being induced in nature all the time and is therefore a source of raw material for natural selection to act upon. In addition, these researchers found that it was changes in nutrition that likely caused the induction of supersoldier-like anomalies in wild colonies (Wheeler, 1902; Goetsche, 1937; Gregg, 1942). As mentioned above, both experimentally induced and anomalous supersoldier-like individuals exhibit a potentially adaptive trait (large size) but also a maladaptive trait (tiny wing vestigial wings). One possible explanation is that not enough time has passed for the re-evolution of mechanisms that can suppress these wing vestigial wings during the development of induced or anomalous supersoldiers. Therefore, in order for such individuals to evolve as a functional subcaste of the colony, there must be selection on large size and on elimination of wing vestigial wings.

We showed that an evolutionary process known as genetic accommodation was responsible for the re-evolution of supersoldiers in *Pheidole*. This process occurs when an environmentally induced phenotype becomes fixed in populations through natural selection (West-Eberhard, 2003). Specifically, genetic accommodation of phenotypes occurs by selecting on genes responsible for increasing the frequency and adjusting the form of a trait. In the case of the re-evolution of *Pheidole* supersoldiers: first, to increase frequency of the supersoldier-like anomalies in the colony, selection occurred on genes that increase the environmental sensitivity for producing supersoldiers, such that supersoldiers become regularly induced by recurrent variation in nutrition or other environmental cues and their frequency increases to approximately 4% of the colony; and second, to adjust the form of supersoldier-like anomalies, selection occurred on genes that eliminate the production of wing vestigial wings. To summarize, Rajakumar et al. 2012 shows that the induction of ancestral developmental potentials, like that which produces supersoldiers, occurs frequently in

natural populations and that they are neither hopeless monsters nor freaks of nature. On the contrary, they are raw materials for natural selection to act upon. This conclusion has important consequences for animal breeding and understanding complex disease as we outline in the following sections.

### **The association between ancestral developmental potential and complex disease in**

**poultry.** Inducing the ancestral developmental potential for supersoldiers not only illuminates the fact that ancestral potentials offer a rich source of raw material for natural selection to act upon, but has also illuminated another important fact – that inducing ancestral potentials in natural populations is often accompanied with the induction of detrimental or negative side consequences (West-Eberhard, 2003; Rajakumar et al. 2012). As we discussed above, we were never able to induce supersoldiers in species that normally lack them without also inducing the appearance of the tiny, but detrimental, wing vestiges (Rajakumar et al. 2012). This observation appears to be generally applicable to other species, including poultry. In the quote at the very beginning of this article, Darwin’s (1868) remark that *“Reversion is most likely the rule, as Mr Sedgwick has shown, with certain diseases...”* indicates that he was well aware of the association between the induction of reversions/atavisms and the appearance of disease. Darwin follows this remark with an example in poultry describing the association between the induction of ancestral potentials and ovarian cancer: *“I will here add a somewhat different case, as it connects in a striking manner latent characters of two classes. Mr. Hewitt possessed an excellent Seabright gold-laced hen bantam, which, as she became old, grew diseased in her ovaria, and assumed male characters. In this breed the males resemble the females in all respects, except in their combs, wattles, spurs, and instincts; hence it would have been expected that the diseased hen would have assumed only those masculine characters which are proper to the breed, but she acquired, in addition well-arched tail sickle-feathers quite a foot in length, saddle-feathers on the loins, and hackles on the neck,—ornaments, which, as Mr. Hewitt remarks “would be held abominable in this breed.” The Seabright bantam is known to have originated about the year 1800 from a cross between a common bantam and a Polish fowl, recrossed en-tailed bantam, and carefully selected; hence there can be hardly a doubt that the sickle-feathers and hackles which appeared in the old hen were derived from the Polish fowl or common bantam; and we thus see that not only certain masculine characters proper*

*to the Seabright bantam, but other masculine characters derived from the first progenitors of the breed, removed by a period of above sixty years, were lying latent in this hen-bird ready to be evolved as soon as her ovaria became diseased.”* This intricate association between the induction of ancestral developmental potential and negative side consequences leading to disease is likely to be the rule and not the exception in poultry.

### **How to use ancestral developmental potentials to select for desirable features in poultry.**

In the previous section, we briefly described the negative role that ancestral developmental potentials can play in animal breeding through its association with negative side consequences leading to complex disease. In this section, we show how ancestral potentials can also play a positive role in animal breeding. After the publication of our article on the role of ancestral developmental potential in the origin and evolution of supersoldiers (Rajakumar et al. 2012), we received a storm of media attention (all you have to do is type “supersoldier ants” into Google for this to become immediately obvious). One of the media posts by Iain Thompson in *The Register* entitled “Boffins hack evolution, create SUPERSOLDIER ANTS: Genetic prestidigitation could engineer new species” makes the following statements about the implications of our findings: *“For example, the aurochs – the massive ancestor to modern cattle that was hunted to extinction by the 1600s – may be recreatable by examining a cow’s genome and finding a way to activate the processes that would cause the much larger and more aggressive aurochs to develop. In the plant world too, crops could be subjected to environmental and chemical stressing to see if the dormant genotypes could be activated. This could usher in new crops that can better deal with current conditions – not to mention changing conditions as climate change wreaks its havoc.”* Could this really be possible? We argue that it is, so long as the focus is on resurrecting specific desirable traits and not whole species. This means that researchers and animal breeders would need to be very familiar with the ancestral traits in the group of interest. The challenge, as we discussed above, will be to find a way to eliminate any negative side consequences that may also be induced. In the following sections we briefly outline two ways that ancestral developmental potentials could be induced in poultry for further artificial selection:

### **Crossing as a tool to release ancestral developmental potential for artificial selection.**

Once again we return to Darwin's ingenious observations in 1868 in *The Variation of Animals and Plants under Domestication*. He recognized that crossing different lineages or species generally induced the appearance of atavistic traits in animals and plants: "...When two races or species are crossed there is strongest tendency to the reappearance in the offspring of long lost characters, possessed by neither parent nor immediate progenitor." He then goes on to give a remarkable example of his observation in poultry: "*I raised several chickens from a Polish hen by a Spanish cock, –breeds which do not incubate, –and none of the young hens at first recovered the instinct, and this appeared to afford a well-marked exception to the foregoing rule; but one of these hens, the only one which was preserved, in the third year sat well on her eggs and reared a brood of chickens. So that here we have the appearance with advancing age of a primitive instinct, in the same manner as we have seen that the red plumage of the Gallus bankiva is sometimes reacquired by crossed and purely-bred fowls of various kinds as they grow old.*" This example beautifully shows that crossing is not only a useful tool for inducing the reappearance of lost physical traits, like plumage, but can also be used as a tool for inducing the reappearance of lost behavioral traits. Indeed, ancestral developmental potential is already being considered key in the generation of behavioral variation and the re-evolution of complex behavioral traits (Foster, 2013).

### **Environmental stress as a tool of releasing ancestral developmental potential for artificial selection.**

The induction of ancestral developmental potential in supersoldier ants shows that environmental factors, such as nutrition, hormones, temperature, and even particular chemicals, can potentially be used as tools to induce ancestral developmental potentials in animals and plants for artificial selection. William Morton Wheeler (1902) and Goetsch (1937) showed that increased nutrition in colonies could give rise to supersoldier-like anomalies in species that normally lack supersoldiers. Because nutrition is so closely linked to levels of particular hormones, it becomes clear why high levels of juvenile hormone could induce the development of supersoldiers in species that lack them. Therefore, it is entirely possible that in poultry, ancestral developmental potential could be induced by alternating sudden increases in nutrition (or hormones) with regular amounts of nutrition in the feed. Temperature and chemical shocks could also be used (Waddington, 1953, Waddington, 1956). For instance, Waddington (1956) performed a classic experiment in fruit flies, where

he applied chemical (ether) to developing fruit fly embryos. Flies by definition only have one pair of wings, whereas all other insects have two pairs. This means that flies lost the second pair of wings during their evolution. The environmental shocks that Waddington (1956) applied induced the reappearance of hindwings, producing adult flies with four wings. Therefore, a range of environmental shocks can be used during poultry development to induce ancestral developmental potentials for artificial selection.

**Fixation of induced ancestral developmental potentials and suppression of negative side consequences through artificial selection.** Although supersoldiers with no wing vestiges took millions of years to evolve, ancestral developmental potentials in poultry can be induced and desirable traits can be fixed through artificial selection in just a few generations. Classic experiments by Suzuki and Nijhout (2006) and Waddington (1956) in insects have demonstrated that ancestral traits induced by temperature or chemical shock, such as pigmentation in caterpillars or the presence of hindwings in fruit flies, can be fixed through artificial selection in as little as 7 generations. Darwin also acknowledged the speed with which induced ancestral potentials can be fixed by artificial selection: *“By the aid of a little selection, carried on during a few generations, most of our cultivated plants could probably be brought back, without any great change in their conditions of life, to a wild or nearly wild condition”* (Darwin, 1868). Therefore, to select for desirable ancestral traits in poultry, breeders should induce ancestral potentials by crossing or by administering environmental shocks each generation, followed by artificial selection. Furthermore, several studies suggest that there are two ways to repress maladaptive traits of induced ancestral potentials: first, studies in chickens suggest that diet regulation, such as calorie-restriction and content, can be manipulated to repress ovarian cancer in chickens (Ansenberger et al., 2010; Carver et al., 2011), and second, studies in insects suggest that artificial selection can be used to simultaneously select positively for desirable ancestral traits while selecting against the appearance of any maladaptive traits (Suzuki & Nijhout 2008). In summary, to use ancestral developmental potentials to enhance genetic lines, poultry breeders should: (1) induce ancestral developmental potentials by crossing different breeds or species of poultry and/or by exposing individuals to environmental shock each generation; (2) artificially select on the induced trait to increase its frequency; and finally (3) repress the

development of maladaptive traits by manipulating diet or selecting against negative maladaptive traits while selecting on positive ancestral traits.

**Conclusion.** We are grateful for the opportunity to have exposed our basic research on the evolution and development of complex societies in ants. We hope to have convinced you or at least provoked discussion on the possibility that ancestral developmental potential is a powerful tool for improving breeding and understanding complex disease in poultry. We maintain that basic research is the fuel of innovation, and that insights gained from the highly organized societies of ants may actually serve as good models for understanding the mechanisms underlying the positive and negative roles of inducing ancestral potentials for animal breeding.

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**Questions for Speaker:      Dr. Ehab Abouheif**

**Question:**      **Dr. Jiuzhou Song**

After induction by the hormone did you check other genes' behavior?

**Answer:**

We focused on the expression of the *spalt* gene as a “read out” of the gene network responsible for wing development because it's position is relatively far downstream in the network and has a unique pattern of expression in the vestigial wing imaginal discs (precursor cells that will develop into the adult wing) of soldier larvae relative to those

queens and minor workers. More importantly, *spalt* expression is associated with apoptosis in the vestigial wing imaginal discs of soldiers, which means that *spalt* may play an important role in eliminating wing vestiges before metamorphosis is complete. We therefore considered *spalt* to be a key gene, which not only serves as a “read out” for the whole network, but also serves to characterize the similarities and differences between castes, including the naturally evolved and induced supersoldiers.

**Question: Dr. Jiuzhou Song**

Is it a single gene or one of gene family?

**Answer:**

*spalt* itself is a single gene, but it is part of the gene network responsible for wing development. Once again, *spalt* expression can be used as a proxy to indicate the overall expression of this network. Future work will have to formally characterize the expression of other genes in this network before and after induction by the hormone.

**Question: Dr. Sue Lamont**

Following on your thought of the positive aspects of “release of genetic variation” by uncovering ancestral potential, what would you speculate as good nutritional manipulations? Methyl donors, to enhance epigenetic changes?

**Answer:**

Methyl donors would be a very interesting group of molecules to manipulate. There is a possibility that supplementing methyl donors in food given to the animals may lead to effects on epigenetic pathways. Since epigenetic pathways (ex: DNA methylation) use folate as a substrate and, along with hormonal pathways, work hand in hand to translate environmental status (ex: nutrition) into effects on development. Therefore, perturbing epigenetic pathways may be as efficient or even more so than perturbing nutrition. On the other hand, perturbing environmental factors like nutrition can induce ancestral developmental potentials, like in the case of ants, where protein has been proposed to be a potential candidate that might specifically be involved in the induction of supersoldiers. That being said, many different nutrients might be involved, and to determine which nutrients are worth testing, we recommend close examination of the diet and natural history of the ancestors of the poultry line of interest.

**Question: Dr. Frank Siewerdt**

Where should we look for clues to explore specific ancestral developmental potential? For example, how could we find out if chickens may lay 3 eggs a day, or sows developing 40 functional teats will one day become a possibility?

**Answer:**

The first question one should ask is “what are their ancestors like?” If their (remote or recent) ancestors exhibited a particular trait, then there is a good chance that the potential to produce that trait is laying dormant in the genome of the contemporary domesticated animal that you are working with. It is also important to note that when ancestral potentials are induced and variation is released, new and different combinations of ancestral traits can arise and be artificially selected for. This process, which is called “developmental recombination” (West-Eberhard, 2003), makes it possible to create new combinations from

ancient ancestral traits. So in theory, chickens that lay 3 eggs a day, or sows developing 40 functional teats may one day become a possibility using ancestral developmental potentials, although it may not be easy. Starting to explore the deep and recent evolutionary history, ecology, development, physiology, and life history of domesticated animals and plants is of primary importance for using ancestral developmental potential to improve animal breeding.

**Question: Dr. Gerald Herbert**

Your talk may infer that “long-term” epigenetic effects and “long-term” multi generational imprinting may play a major role in evolution. This contrasts with the concept of mutations in DNA increasing and decreasing in frequency, (changes in gene frequency) as the mechanism underlying evolution. Evolution may change partially due to activation and inactivation of genes by environmental influences.

**Answer:**

Your last sentence elegantly summarizes the general implications of our work. However, it is currently thought that although it is indeed the perturbation of hormonal pathways or epigenetic mechanisms may facilitate the initial emergence of an ancestral phenotype following an environmental induction, it is the fixation of standing genetic variation or *de novo* genetic mutation that preserves the induced phenotype across generations. However, it is entirely possible that trans-generational epigenetic imprinting may be a mechanism which permits the environment to have a persistent effect across generations, which is only subsequently followed by the “genetic fixation” of the initial imprint. Clearly, much research remains to be done in this new and exciting area of research.

# The Current and Future of Epigenetics of Marek's Disease in Chickens

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## Abstract

Epigenetic regulation in host-pathogen interaction is important in the etiology of Marek's disease (MD). These epigenetic regulatory mechanisms, including DNA methylation and histone modifications, are influenced by Marek's disease virus infection. To facilitate epigenetic strategy and methods in animal health, it is of interest to investigate how and what the new epigenetics and genetics influence the infectious disease. With this aim, we summarize the current state of knowledge in order to provide insight in this emerging epigenetic field. We also present the evidence for epigenetic effects for MD control.

## Introduction

Marek's disease (MD) is a lymphoproliferative disease in chickens caused by Marek's disease virus (MDV) and characterized by T cell lymphoma. The MDV is a naturally oncogenic, highly contagious, and cell associated alpha-herpesvirus (NIIKURA *et al.* 2007). The disease is characterized by a mononuclear infiltration of the peripheral nerves, gonads, iris, various viscera, muscles, and the skin. Partial or complete paralysis is a common symptom of MD due to accumulation and proliferation of tumor cells in peripheral nerves. During the 1960s, as the industry converted to high-intensity rearing, MD generated tremendous economic losses. Since the 1970s, MD has been controlled by vaccination. However, although vaccination prevents the formation of lymphoma and other MD symptoms, it does not prevent MDV infection, replication, or horizontal spread (PURCHASE and OKAZAKI 1971). Thus, to advance MDV evolution escalating to higher virulence, knowledge supporting development of etiology of MD and new strategies for control of MD need to be developed. For a long time, resistance to MD and disease risk have long been thought to be influenced by genetic, environmental factors, and the combination of which contributes to the observed outcome in an individual. To augment vaccination measures, host genetic resistant to MD becomes obviously more and more important. To control MD efficiently, we need to further explore the mechanisms of host-virus interactions. However, most of researches focused on the genetic differences between resistant and susceptible chickens to elucidate

the mechanisms of MD resistance (KAISER *et al.* 2003; SARSON *et al.* 2008). The more details of host-MDV interaction are not understood. In our research, we believe that the interaction is an intrinsic epigenetic mechanism and needs to be characterized thoroughly. The advancement of the host-virus interactions will help us understand the etiology of MD and facilitate epigenetic methods in resistance to MD, which, in turn, should result in a deep insight on disease resistance breeding in chickens.

### **DNA methylation analysis in Marek's disease**

Epigenetics involving phenomena such as DNA methylation, chromatin modifications and non-coding RNAs constitutes a dynamic regulatory framework linking genotypes with environmental factors in differential disease responses among individuals having high genetic similarity (MITRA *et al.* 2012). Although several efforts have attempted to pinpoint factors responsible for conferring MD resistance (LIU *et al.* 2001; WAIN *et al.* 1998), many confounding factors, such as, tissue types, virus strains and ages of birds have made it difficult to find a consensus for the complex disease. In our studies, therefore, we took epigenomics approach in highly inbred lines to identify the mechanisms that contribute to the neoplastic diseases by utilizing the tractable and powerful combination of DNA methylation, histone methylation, microRNAs, statistical genomics and computational methods. The line 6<sub>3</sub> at the Avian Disease and Oncology Laboratory (ADOL) is relatively resistant to MD tumors but is susceptible to Marek's disease virus (MDV). However, another line 7<sub>2</sub> is susceptible to both MDV and MD tumors (BACON *et al.* 2000). Therefore, these inbred lines with high degree of genetic similarity constitute unique models for epigenetic research because they make it possible to explore mechanisms of resistance and susceptibility to neoplastic diseases.

Methylation of cytosines at CpG dinucleotides is an epigenetic modification of DNA that has been shown to play important roles during tumorigenesis, embryonic development, and X chromosome inactivation in mammals (MORGAN *et al.* 2005). The function of DNA methylation is further heightened by the association of epigenetic dysfunction with various diseases, in particular neoplastic diseases. Generally, DNA methylation is known as a post-replication modification generated and maintained by three methyltransferases-DNMT1, DNMT3a, and DNMT3b (KOROCHKIN 2006). Notably, we found two DNA mutations in *DNMT3b* (Yu *et al.* 2008b) and a higher promoter methylation level of *ALVE* and *TVB* in the

spleen of MD-susceptible chickens (L7<sub>2</sub>) compared to that of MD-resistant chickens (L6<sub>3</sub>) (Yu *et al.* 2008a), and the methylation level in *CD4* promoter region was down regulated in the former but not in the later at 21dpi (Luo *et al.* 2011). To advance the understanding of functional patterns of DNA methylation in disease resistance or susceptibility, we extended the scope of examination to interested genes (Luo *et al.* 2012b), which include genes related to immune that the expression levels of these genes are alterable upon MDV challenge (HEIDARI *et al.* 2010; THANTHRIGE-DON *et al.* 2010). We found DNA methylation heterogeneity between the MD-resistant L6<sub>3</sub> and –susceptible L7<sub>2</sub> chickens. Since MDV induces a dynamic expression change in *DNMTs*, differential methylation changes have been observed between resistant and susceptible chickens after MDV infection. To thoroughly ascertain the methylation variation in induced by MDV infection in both chicken lines, we mapped the genome-wide DNA methylation profiles in each line using Methyl-MAPS (Methylation Mapping Analysis by Paired-end Sequencing). We found that the methylation levels were reduced in chickens from the resistant line 6<sub>3</sub> after MDV infection and 11,512 infection induced differential methylation regions (iDMRs) were identified. The number of iDMRs was larger in line 7<sub>2</sub> than in line 6<sub>3</sub>, and most of iDMRs found in line 6<sub>3</sub> were overlapped with the iDMRs found in line 7<sub>2</sub>. Importantly, we further demonstrated that *in vitro* methylation levels were associated with MDV replication, and found that MDV propagation in the infected cells was restricted by pharmacological inhibition of DNA methylation. The results suggested that DNA methylation changes in the host may be associated with disease resistance or susceptibility. The methylation variations induced by the viral infection may consequentially change the host transcriptome and result in diverse disease outcomes. All in all, the differential DNA methylation levels and its change induced by MDV challenge between the lines of chickens suggested that DNA methylation may play a role in host resistance and/or susceptibility to MD (LUO *et al.* 2012b; TIAN *et al.* 2013).

### **Histone modifications in Marek's disease**

Although our study found that MD-resistant and susceptible birds with different DNA methylation levels on several candidate genes, indicating the potential functions of epigenetic factors in inducing different tumor incidence rates. However, little was known about the histone modification patterns in these two chicken lines before. Therefore, to

gain more insight into the function of histone modifications in MD, we performed a histone landscape analysis using ChIP-Seq in the unique MD-resistant (L6<sub>3</sub>) and –susceptible (L7<sub>2</sub>) chicken lines both before and after MDV infection. Large number of line-specific H3K4me3 modifications and their underlying genes in immune response and cell adhesion in L6<sub>3</sub> chicken were found. Interestingly, we also found that the virus-induced specific H3K27me3 patterns in L7<sub>2</sub> chicken overlapped with some miRNAs which target genes involved in novel pathways that may be related to MD-susceptibility (Luo *et al.* 2012a). Besides, WaveSeq, a novel data-driven method of detecting regions of significant enrichment in ChIP-Seq data was developed. The distribution-free method utilizes the wavelet transform, and is robust to diverse data characteristics such as low signal-to-noise ratios and broad enrichment patterns. The WaveSeq can detect both narrow and broad peaks with a high degree of accuracy even in low signal-to-noise ratio data sets, and it is also suited for application in complex experimental scenarios, helping make biologically relevant functional discoveries (MITRA and SONG 2012).

### **Gene Expression Analysis to Marek's Disease**

Natural resistance to MDV can be divided into two categories: major histocompatibility complex (MHC)-associated resistance, wherein different MHC haplotypes at the B blood group locus confer varying levels of resistance and non-MHC associated resistance in which birds having the same MHC haplotype exhibit vastly different responses to MDV infection. Inbred lines 6<sub>3</sub> and 7<sub>2</sub> we used in this study fall into the latter category. These lines share a high degree of genetic similarity but have divergent responses to MDV infection completely independent of the MHC. Earlier reports have shown that the MHC haplotype as well as non-MHC genes are responsible for genetic resistance to MD (BACON and WITTER 1993; BACON and WITTER 1994; BACON and WITTER 1995). To further explore transcriptome differences after MDV infection, besides significant expressed genes, we also identified other non-coding RNAs during the development of MD and screened miRNAs that were sensitive to Marek's disease virus (MDV) infection. Our results indicated that differential expression of miRNA in resistant and susceptible chickens was caused by MDV infection, which effectively influenced protein expression of ATF2 that might be related to Marek's disease resistance/susceptibility (TIAN *et al.* 2012). The results above will facilitate the discoveries of etiology mechanisms and chromosome loci which convey MD resistance.

### **Genetic Variations to Marek's Disease**

Aside from epigenetics, genetics and environmental factors are also causative agents to MD. Some studies indicated that genetic variations are associated with the susceptibility of MD (EMARA *et al.* 2001). To identify new genetic markers, we used high density SNP chips to screen potential genetic markers and detect CNVs in the chicken lines. Besides, the DNA mutations of DNA methyltransferases (DNMT3a, DNMT3b and DNMT1) were found and their relationships with genes expressions of themselves were analyzed (Yu *et al.* 2008b). Meanwhile, the complexities and structural characteristics of these genes in entropy view were analyzed to help us elucidate mechanisms and effects of the genes in epigenetic processes (XIE X 2010). To have a full understanding of vaccination in MD control, we also examined the effect of host genetic variation on vaccine efficacy (CHANG *et al.* 2010) and comparative evaluation of MD virus infection in a series of recombinant congenic strains (CHANG *et al.* 2011). In these studies, the lipoprotein metabolism in Marek's disease susceptible and resistant chickens was characterized (YUAN P 2012). All of these are around the Marek's disease to expose its real biological mechanism.

### **Future of epigenetics of Marek's Disease**

Epigenetics is an active and exciting area of research. It is being driven by the massive amounts of new information being generated by next generation sequencing methods. Current epigenetics offers perhaps the greatest potential for animal health. Although we don't understand the mechanisms, we will identify unique epigenetic factors that could be potentially used as epigenetic biomarkers. The knowledge of host-pathogen interaction will provide a better understanding of epigenetic modifications at a 'systems level' and will serve as mechanistic studies aimed at defining epigenetic roles that underlie disease resistance. Most importantly, we believe an improved strategy for epigenetically preventive measures against disease will subsequently pave the way for more focused and efficient application of marker-assisted selection (MAS) or genomic selection in poultry breeding program in the near future.

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# **Estimation of genetic parameters for behavioral assessment scores in Labrador Retrievers, German Shepherd Dogs and Golden Retrievers.**

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## **The Guide Dog Service Industry**

The use of dogs guiding blind people is speculated to date as far back as the Pleistocene Age (Coon, 1959) and today, guide dogs are ranked as one of the most noble and useful occupations of all working dogs (Willis, 1995). To keep pace with increasingly more complex working environments where guide dogs are asked to assist their blind masters, guide dog breeding programs must constantly improve the working ability and general health of the puppies they produce. Two guide dog schools in the U.S., Guiding Eyes for the Blind and The Seeing Eye, Inc., have undertaken this challenge by setting the ultimate goals of their breeding programs to increase the number of healthy, high-quality dogs available for training while minimizing the number of dogs released.

Guiding Eyes for the Blind Headquarters and Training Center is located in Yorktown Heights, New York, while their Canine Development Center (Breeding Center) is located in

Patterson, New York. Guiding Eyes for the Blind is an internationally accredited guide dog school with a mission to provide greater independence, dignity, and new horizons of opportunity to the visually impaired. Their selective breeding program began in the mid-1960s and the organization continually strives to genetically improve the health and working ability of its guide dogs.

The Seeing Eye, Inc., located in Morristown, New Jersey, was founded in 1929 and is the oldest existing guide dog school in the world. It is an internationally accredited provider of Seeing Eye® dogs and owns the trademark for and is technically the only source for a dog with this designation. The Seeing Eye, Inc. is an international philanthropic organization, providing Seeing Eye® dogs to blind people in both the U.S. and Canada. It is renowned as a research leader in canine genetics, breeding, disease control, and behavior. Their overall mission is to enhance the independence, dignity, and self-confidence of blind people through the use of Seeing Eye® dogs.

In addition to Guiding Eyes for the Blind and The Seeing Eye, there are at least 8 other schools in the U.S. that provide dogs as trained guides for blind people. It is estimated that about 10,000 blind people in the U.S. use a guide dog as part of their daily walk through life.

### **Breeding and Selection of Guide Dogs**

Obtaining an adequate number of high-quality dogs suitable for breeding or training to work as guides for blind people is a constant challenge for many guide dog schools. To meet their needs, many schools now maintain their own breeding colonies to ensure an adequate supply of high-quality dogs with predictable traits. The most effective way to use information about the traits in a population, to produce predictable stock and to improve it over time, is to apply the time-proven principles of population genetics and quantitative genetics to manage the population over successive generations (Bourdon, 2000).

Over the past three decades, advances in computing power have enabled the study of genetic complexity in more animals and in greater detail than ever before. Researchers can now predict changes in quantitative genetic traits in future generations by using complex statistical models that take into account all the animals in the pedigree. Several traits can be studied simultaneously and relationships among traits can be established.

Most parameters of behavior are complex combinations of genetic and environmental influences. With sufficient data on each of many animals in an extended pedigree, it is possible to assess the extent to which these traits are heritable. Furthermore, researchers can also forecast the likely results of selection in the future, which enables comparisons of the possible results of different breeder selection strategies (Bourdon, 2000).

One of many issues facing guide dog organizations is that there is no single perfect “type” of guide dog. Because substantial variation exists among the people who use guide dogs, there must also be variation among the dogs available for matching with their blind masters. This means that guide dog schools are not breeding for one specific kind of "super dog", as show breeders predominantly desire. Rather, it is essential to have dogs with varying degrees of characteristics such as breed, temperament, size, and energy to enable guide dog schools to properly match dogs with blind people who are equally varying in their needs and desires. After all, the dog with which they are ultimately matched will literally live with them 24 hours per day.

This task of finding the “right” dog for each client is one of the hardest jobs faced by the training staff of a guide dog school. The trainer has to assign the dog to the client/handler early on in the training course as they have a short period of time with the clients and dogs together to determine if they have made a successful match. It is very difficult to switch a pair late in the training period as bonding between the client and dog will already have begun to occur. Each trainer works with a small group of clients and dogs at the same time and therefore has potential to create confusion for the dog when reassigning them with a different client, but having to remain around their original handler. It is also important that the dog’s temperament matches the home environment of the client. For example, it would be unwise to pair a high energy large dog with a short elderly client who doesn't often leave their home.

Behavior issues top the list of most common reasons for rejecting dogs from working as guides (Goddard and Beilharz, 1982, 1982/83). The criteria used in the breeder selection process at guide dog organizations are crucial to the success of working guides. A potential tool for this selection process is known as the Canine Behavioral Assessment and Research Questionnaire (C-BARQ, 2012). The C-BARQ was developed by researchers at the Center for the Interaction of Animals and Society at the University of Pennsylvania and was designed to provide standardized evaluations of canine behavior and temperament. Information the C-

BARQ provides may be useful to guide dog schools as a way to learn more about the nature of optimal behavioral traits leading to the production of dogs that are more successful in their ability to work as guides.

The C-BARQ was validated in two separate, but similar studies (Serpell and Hsu, 2001; Hsu and Serpell, 2003) confirming that the questionnaire factors and the behavior and temperament traits they represent were stable and consistent among different populations of dogs. The authors (Serpell and Hsu, 2001) also evaluated the overall construct validity of the C-BARQ, which was deemed valid as well as capable of discriminating among dogs that succeed in training versus dogs that fail training for behavioral reasons. Complete details describing the process used to validate the C-BARQ and assess its reliability have been reported (Serpell and Hsu, 2001, 2005; Hsu and Serpell, 2003).

While this canine behavioral assessment instrument has been extensively tested for reliability and validity, prior to this ongoing research a comprehensive genetic analysis of the C-BARQ had not been completed. Thus, the objectives of this research were to estimate the magnitude of heritability for each of the 101 questions and each of the 12 subscale factors, as well as exploration of breeding strategy implications based on the results obtained.

### **Current Research Results**

At 6- and 12-months of age respectively, questionnaire responses were obtained on 3,149 and 3,348 Labrador Retrievers from Guiding Eyes for the Blind and 989 and 1,187 Labrador Retrievers, 608 and 692 Golden Retrievers, and 966 and 1,348 German Shepherd Dogs from The Seeing Eye, Inc. The C-BARQ questionnaires were completed by the puppy raiser families, who as volunteer families, raise these puppies in home environments from about 7-8 weeks of age until they return to the school to begin training at 14-18 months of age. He/she is asked to rate his/her dog's typical response to a range of commonly encountered events, situations, and environmental stimuli (Serpell Hsu, 2001, 2005; Hsu and Serpell, 2003).

The estimates of heritability and standard errors from The Seeing Eye, Inc. dogs indicate that there is much genetic variation that could be exploited in selection against "Familiar dog-directed aggression/fear" of Golden Retrievers at 6-months, "Chasing" of

Golden Retrievers at 6-months, and “Nonsocial fear” of Golden Retrievers at 12-months or in selection for improved “Trainability” of Labrador Retrievers, German Shepard Dogs, and Golden Retrievers at 12-months. Heritability values obtained from the current study can be used as a guideline to approximate the outcome of various selection decisions (Willis, 1995). Furthermore, some of the subscale factors identified as being moderate or highly heritable might also prove useful for predicting which puppies may be at risk of failing from the guide dog program. In general, the remaining factors and most of the 101 questions were found to be lowly heritable (< 0.10). These estimates are useful to understand more about the nature of behavioral traits leading to the production of successful working guides and provide fodder for future research.

### **Future Research**

There is a great need to develop a common language in describing temperament traits of the dog to enable comparisons across populations. Estimating the direction and magnitude of genetic correlations between the subscale factors would be beneficial to determine if any two traits are influenced by common genes. The C-BARQ may also be a potential tool to predict which puppies may be most at risk of failing from the guide dog program as well as determining more specifically how the scores relate to success.

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**Questions for Speaker:     Kelly Schulz**

**Speaker:     Kelly Schulz**

**Question:**     **Dr. Sammy Aggrey**

Would you be willing to reanalyze your data using categorical trait model?

Yes, that is my current plan.

**Question From:**     **Dr. Jesus Arango**

Q1). Sex was the fixed effect in the MME: Did you find differences between sexes for any of the analyzed traits?

Yes.

Q2). You analyzed traits at two ages; did you carry bi-variate analyses to explore genetic correlations between traits?

It is in our current plans to do so for the subscale factors across both time points.

Q3). You had long pedigrees for both populations; did you estimate inbreeding and change of inbreeding in these populations?

**Answer:** No, as both organizations calculate their respective inbreeding coefficients and monitor it continually.

**Question From:**     **Dr. N Matthew Ellinwood**

**Question:** Did you incorporate a dam effect in your model?

**Answer:** No, it was not found to be statistically significant in the preliminary analysis.

For more details, please visit: <http://krex.k-state.edu/dspace/bitstream/handle/2097/13660/KellySchiefelbein2012.pdf?sequence=1>

# Genetic Modification of Pigs: Expanding their Utility as Biomedical Models

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## Introduction

Development of transgenic animals has become technically more feasible over the past several decades creating opportunities for the genetic modification of numerous species. This dramatic expansion in the number of genetic modifications in pigs, in particular, is due to the advancement of molecular techniques that facilitate genetic modification as well as their agricultural importance and physiological characteristics, which make them an ideal model to conduct translational biomedical research. Genetic modification of pigs can address both basic and applied research questions with implications for agriculture and medicine.

## Pig Biomedical Utility

Pigs are increasingly being utilized as biomedical models. Even without genetic modification, pigs have been used by many investigators to model cardiovascular physiology, reproduction, skin physiology, respiratory function and infectious disease (Lunney 2007; Prather et al. 2008; Yang and Ross 2012). However, precise manipulations of pig genomes allows the generation of pig models for specific genetic diseases. Strategies for introducing genetic modifications into the swine genome have accelerated their utility as biomedical models by facilitating rapid development of numerous transgenic pig models. Multiple approaches have been explored to create transgenic animals that have been previously characterized with respect to the strengths and weaknesses of each approach (Ross and Prather 2010; Yang and Ross 2012).

## Strategies for Developing Transgenic Pigs

Milestones achieved in the development of transgenic pigs often follows shortly after similar achievements in mice. For example, the first transgenic mice were developed over 30 years ago (Brinster et al. 1981; Costantini and Lacy 1981; Gordon et al. 1980; Wagner et al. 1981) and the technique was further refined to include utilization of

embryonic stem (ES) cells coupled with homologous recombination to create mice with targeted genetic modifications (Doetschman et al. 1987; Thomas and Capecchi 1987).

Pronuclear injection of linearized DNA into the single cell zygote pronuclei, however, is not an efficient process. Nevertheless, not long after publication of the first knockout mice, the technique was used to produce transgenic pigs (Bleck et al. 1998; Hammer et al. 1985; Petters et al. 1997; Vize et al. 1988) and other large animal models (Bondioli et al. 1991; Wang et al. 2002). Sperm mediated transgenesis (SMTG) relies on the ability of mammalian sperm to bind DNA consequently serving as the vehicle through which exogenous DNA constructs can be delivered into the newly created zygote. SMTG can occur via insemination or intracytoplasmic sperm injection (ICSI) (Kurome et al. 2007; Kurome et al. 2006; Lavitrano et al. 1997; Pereyra-Bonnet et al. 2008; Perry et al. 1999; Webster et al. 2005). In the case of oocyte transduction, AAV vectors integrate into metaphase chromosomes while the oocyte is arrested in metaphase II of meiosis. The strategy was first accomplished in cattle (Chan et al. 1998) and later, a similar approach in pigs was used to create the first eGFP transgenic pigs (Cabot et al. 2001).

While those strategies have been useful in creating transgenic pigs, the most commonly utilized approach is the genetic modification of a somatic cell followed by nuclear transfer. Since the birth of Dolly in 1996 (Wilmut et al. 1997) somatic cell cloning of other large animal species (Baguisi et al. 1999; Cibelli et al. 1998; Galli et al. 2003; Jang et al. 2007; Woods et al. 2003; Yin et al. 2008) (Oh et al. 2008), including pigs (Polejaeva et al. 2000) has been widely used.

### **Genetic Modification followed by Somatic Cell Nuclear Transfer**

Generation of cloned transgenic pigs through nuclear transfer requires the utilization of a donor cell whose genome can be effectively remodeled and reprogrammed to complete term gestation. Importantly, that cell type must be capable of being genetically modified prior to SCNT. While numerous cell types in pigs have been successfully used (Beebe et al. 2007; Brunetti et al. 2008; Hornen et al. 2007; Hyun et al. 2003; Kurome et al. 2008; Lai et al. 2002a; Lai et al. 2002b; Tomii et al. 2005), we have had success primarily with fetal fibroblasts (Ross et al. 2012; Ross et al. 2010; Zhao et al. 2009). Fetal fibroblasts are used as they generally do not senesce until after ~30 days making genetic engineering and selection within their lifespan feasible. Efficient generation of genetic modifications in donor cell

genome relies not only on the targeting/integration strategy but also on the efficient introduction of the exogenous DNA into the potential donor cell and subsequent selection of stable integrated cells. In pigs, a variety of methods have been quite effective at introducing exogenous DNA into the somatic cell, including lipid based delivery (Hyun et al. 2003; Lee et al. 2005), viral delivery (Rogers et al. 2008), and electroporation (Arat et al. 2001; Ramsoondar et al. 2003; Ross et al. 2010). These strategies have been used by us and others to create genetically modified pigs that are contributing to biomedical research (Prather et al. 2013).

## **Specific Models**

### *Retinitis Pigmentosa*

Using some of the above mentioned approaches we have developed a miniature swine model of retinitis pigmentosa (RP) (Ross et al. 2012). In this case, we utilized the human rhodopsin gene containing the P23H mutation, the most common mutation in the rhodopsin gene resulting in RP in humans. The construct was linearized, and electroporated into the cells using the strategies we developed for transfection of pig fetal fibroblasts (Ross et al. 2010). The result was the production of six founder P23H miniature pigs. Each of the founders demonstrated a unique disease phenotype with respect to disease onset and progression. These differences in phenotype are likely the result of copy number variation of the integrated transgene and the different integration sites in the genome between the founders, as demonstrated by in situ hybridization and southern blot analysis. This model is now being utilized as a translational research model for investigators working towards developing strategies to mitigate the devastating effects of RP in humans.

### *Muscular Dystrophy*

While genetic modifications to pigs can be made to create novel and useful biomedical models of disease, some can be serendipitously discovered, as was the case with the discovery of a pig model of Becker muscular dystrophy (BMD) by a research group at the USDA and its subsequent characterization. BMD, caused by a dystrophin insufficiency, is a progressive muscle disease where over time whole muscles become more fibrotic and less functional. It is related to the more severe Duchenne muscular dystrophy (dystrophin deficiency), which is a 100% fatal disease due to respiratory or cardiac failure in the mid-20s. BMD patients may have disease phenotype that closely matches that of DMD or a milder

phenotype, depending on the causative mutation. A line of pigs that died at a high rate during transport was found to have a mutation in the dystrophin gene and a 60-90% reduction in dystrophin protein abundance (Nonneman et al. 2012). In conjunction with this group, we found recently that this mutation led to muscle injury in the diaphragm and longissimus but not the psoas at only eight weeks of age. This is important because the diaphragm and longissimus are used far more frequently than the psoas in penned animals pointing toward a use-dependent disease severity. Also, dystrophin insufficiency led to a failure of the dystrophin-glycoprotein complex to correctly assemble, which is also consistent with the disease observed in humans. Our next step will be to determine the extent to which dystrophin insufficiency leads to muscle injury and loss of muscle function as the disease progresses.

## Conclusion

Pigs are and will continue to be utilized as biomedical research models. Development of strategies to improve the efficiency of genetic modifications, such as the utilization of zinc finger nucleases and Tal effector nucleases will further enable precise genetic manipulation of pigs to expand their utility as biomedical models (Carlson et al. 2012; Whyte and Prather 2012).

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## **White Striping in Broiler Breast Meat**

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### **Summary**

White striping is an emerging issue in the broiler meat industry which causes concerns among the producers. The condition is characterized by the occurrence of white striations, parallel to the direction of muscle fibers, in broiler breast fillets. Varying levels of severity can occur. The present work reviews studies that determined the consumer acceptance, meat quality and pathological changes associated with different degrees of white striping. The occurrence of moderate and severe degrees was associated with heavier birds or increased growth rate in broilers. Serologic profile and the histopathological findings indicated chronic myopathic tissue changes associated with higher degrees of white striping along with an increase in fat. White striping in fillets reduced the consumer acceptance of the visual appearance of breast fillets, but did not have major effects on meat quality. White striping is a growth associated myopathic condition seen in fast growing broilers resulting in a product defect.

### **Introduction**

Boneless broiler breast meat is a popular product in the global poultry industry. It is basic form of adding value, resulting high value cuts. Broiler breast meat is used in a variety of markets including retail, further processing, and foodservice. Though variation around the world can exist in the amount of broiler breast meat on the market, it is or will be a leading product, depending on the country. Due to the popularity of boneless breast meat, processors have been using large, high breast yielding broilers as their source in recent years. The high yielding birds in the big bird market allows for increased yields and pounds per man hour in processing. In the U.S., the average live weight of birds in this segment is now approximately 3.4 kg, but can range from 2.7 to 4 kg or more. The big bird market (>2.7 kg) segment makes up over 40% of the overall market in the U.S. with approximately 18% being birds 3.7 kg. This trend for increased bird size and the increased percentage of

the market will likely continue. Like the U.S., in other countries, broilers are also growing at a fast rate with high breast yields.

One issue that has become more apparent in recent years in the U.S., Brazil, and the overall global poultry market is the appearance of white striations in the breast muscle, typically in larger broilers (Kuttappan et al., 2009; Kuttappan et al., 2012; Ferreira, 2012). The appearance of white striations in muscle has also been commonly referred to the condition called *white striping*. White striping is a condition characterized grossly by the appearance of white striations, parallel to the direction of the muscle fibers, mainly on the ventral surface of broiler breast fillets. It can also be observed in thighs and tenders. The severity of the condition can vary from none (normal) to severe. Kuttappan et al., (2012) developed a classification system based on visual appearance. Fillets have been classified into multiple categories including normal (NORM), moderate (MOD), or severe (SEV) based on the severity of the condition. However, more extensive categories can also be used if needed.

This condition is an emerging problem in the poultry industry, worldwide, and therefore, the research associated with it is relatively recent as well. Recent research in our lab, has been conducted to evaluate various aspects associated with the condition. The objectives have been to characterize the condition, evaluate meat quality and sensory attributes, and to determine the causes and/or influencing factors.

### **Description of the Condition**

The condition is characterized by white striations, or “stripes,” that run parallel to the muscle fibers often beginning at the cranial portion of the fillet near the wing attachment where it is most concentrated. The striping is readily visible even in moderate cases across the fillet, but can be distracting in severe cases where the lines are very prominent. As the condition worsens as in severe cases, the stripes, or striations, are visible throughout the fillet (length of muscle fibers and from the cranial to caudal portion of fillet) and can become wider in appearance. Other reports from industry show that in extreme cases, the ‘white’ portion can cover a much greater area (i.e., not only a striation). While this may be less common at the time, the industry could potentially see these extreme cases more frequently in the future as the industry continues to make advancements in broiler performance.

Upon histological examination, the striped areas of the fillet are characterized by an increase in fat cells (lipidosis) and connective tissue (fibrosis) along with areas of muscle fiber degeneration. It is likely that fat and connective tissue infiltrate the areas where fiber degeneration occurs (Kuttappan et al., 2009; 2013b). It has been suggested that the condition is a form of muscular dystrophy with unknown causes. Analysis of serum enzymes showed that SEV fillets has elevated levels of enzymes related to muscle damage (Kuttappan et al., 2013a) supporting the histological findings. Furthermore, evaluation of proximate analysis show increased fat percentage which also supports the histological analysis (Kuttappan et al., 2012a, 2013b). The histological and proximate changes are more associated with the ventral surface location of the fillet (Kuttappan et al., 2013b). Kuttappan et al (2013a) evaluated hematologic profiles of NORM and SEV birds and results suggested that white striping was not a result of any infections, inflammatory or stress condition.

### **Body Size and Growth Rate**

Previous studies and communication with the industry indicate that the condition is associated with fast growth rates and heavy birds (high breast yielding broilers). In a recent study, high incidences (>50%) of white striping were observed when processing broilers at approximately 60 d of age (Kuttappan et al., 2009). Other studies have shown that the incidence of white striping in breast increases when birds are processed at older ages and/or heavier weights (e.g., 8 wk vs. 6 wk) (Bauermeister et al., 2009). Bauermeister et al. (2011) also reported that birds with higher breast meat yield exhibited a higher incidence of striping compared to birds with lower breast meat yield. Furthermore, males tend to have a greater incidence of striping (or severity of) than females, but this is likely due to body and fillet size differences (Kuttappan et al., 2009). Though the condition is associated with heavier broilers, it does not seem to be strain specific. Differences that may exist among strains are likely to occur due to different growth rates/breast yields associated with the various strains. Growth rate has also been evaluated and a faster growth rate has been associated with a higher incidence of birds exhibiting white striping (Kuttappan et al., 2012a). Trends in the industry over the past few decades have been to produce a broiler with a typical market weight in a shorter period of time (i.e., faster growth rate). Since 1925, the average market weight has more than doubled (1.1 to 2.6 kg) in less than half the growout time (112 d to 47 d) (NCC, 2011). Breast meat yield has also increased significantly during that period (Havenstein et al., 2003). More recent trends in the poultry meat

industry are to produce birds to heavier weights (thus, older birds) in order to gain more breast meat per bird for portioning and further processing. Therefore, it is likely that a high percentage of today's birds will be affected by moderate or severe white striping now and in the future. Also, a percentage of birds may exhibit severe or extreme cases of this condition more frequently as well.

### **Meat Quality**

To date, general meat quality attributes have not been affected by the white striping condition. Kuttappan et al. (2009) evaluated water holding capacity, color and tenderness and reported no differences in those attributes that would affect eating quality. However, when evaluating fillet dimensions, breast fillets exhibiting the white striping condition were also larger in width and height compared to normal fillets (Kuttappan et al., 2009) which is expected since this condition is associated with larger broilers, or those with a rapid growth rate. Due to the limited research available, further research is still warranted in this area especially to evaluate the most extreme cases of severity. The condition could potentially lead to issues with product quality in the future.

### **Appearance**

Because white striping is visually apparent on raw breast fillets, the consumers evaluated the visual appearance of raw breast fillets and determined acceptability. Kuttappan *et al.* (2012b) showed that consumer acceptance of raw fillets significantly decreased as severity increased. In that study, a high negative correlation between image analysis of 'white area' and consumer hedonic score was noted. This suggests that image analysis could be used for future quality control measures. It was also reported that consumer purchase intent significantly decreased when any degree (moderate or severe) of white striping was apparent. The major reason for the consumer dislike, indicated by consumer comments, was the "fatty" or "marbled" look of the product. These findings are important because consumer acceptance of products can have a major impact on their purchasing choices. In the U.S., fresh, raw breast fillets are common in the retail market (i.e., grocery store) and size of fillets can vary between specific products and brands, coming from birds from a wide variety of sizes (e.g., 22 to 3.6+ kg). Broiler breast meat is also commonly exported from Brazil where this is also a quality issue (Ferriera, 2012).

### **Future Outlook**

There are still no known causes for the condition other than its relationship to growth rate. It has been suggested that the causes may be related to nutritional deficiencies, but this has not been fully determined yet. Kuttappan et al. (2012c) evaluated the effect of various levels of vitamin E, ranging from 15 to 400 IU of vitamin E/kg of feed, and determined that dietary vitamin E had no effect on the incidence of moderate or severe striping in broilers. More research is needed to determine other dietary factors that may be involved.

Regardless of the cause, processors have to deal with the problem now. For product going into the raw retail market, it would be advantageous for processors to sort the product based on striping severity. Generally, fillets categorized as severe have stripes that are very prominent and distracting to the eye. Additionally, using image analysis could potentially be a tool for sorting fillets though the methods would have to be optimized. Regardless, fillets exhibiting the most severe striping should be diverted away from products that are sold as raw where consumers are able to choose product based on appearance. The white striping condition does not appear to be an issue in cooked fillets based on appearance or meat quality. Therefore, fillets with severe striping may be used for other products such as further processed, pre-cooked fillets or breaded products (whole muscle or comminuted). However, research is needed even in the area of further processing and the incorporation of striped fillets into products. It is possible that in the severe cases, product quality may be affected.

There is still a great deal of research that is needed in this area. This condition has been observed over the past several years and with the way birds are marketed today (e.g., large, high breast yielding), it is likely that white striping in broiler meat will continue to be an issue.

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**Questions for Speaker:**        **Dr. Casey Owens Hanning**

**Question From:**        **Dr. Gerald Herbert**

**Question:** Do you feel controlling growth by means of a suitable lighting program (light restriction) would help control the expression of the condition?

**Answer:** Increased growth rate (especially in high breast yielding birds) has been shown to result in increased incidence. While no research has been conducted to evaluate lighting programs to determine its effect on white striping, it is plausible that slowing growth by controlling light programs could reduce the incidence, or severity of. However, desired body weights would not be achieved in same period of time thereby affecting efficiency. It would be a trade-off to consider.

**Question From:**        **Dr. Marco Quiroz**

**Question:** What is the average incidence of white striping in a commercial 7 lbs flock?

**Answer:** Some degree of white striping is common in flocks of this size, based upon personal observations. Moderately striped fillets would be more prevalent than severely striped fillets though it is likely there would still be some prevalence of severely striped fillets. However, no study has been conducted in the US to determine incidence in the marketplace (as opposed to research settings). In Italy, Petracci et al. (2013) recently surveyed 56 flocks (28,000) broilers (approx. 6 lb liveweight) and reported up to 26% in some flocks (moderate and severe degrees) with the average over all flocks at 12% (8.9% moderate and 3.1% severe). It is safe to assume that if broilers were processed at a larger weight, the % incidence would increase. Petracci also reported that the high breast yielding strains had a higher incidence of striping compared to standard yielding broilers. Petracci, M., S. Mudalal, A. Bonfiglio, and C. Cavani. 2013. Occurrence of white striping under commercial conditions and its impact on breast meat quality in broiler chickens. *Poult. Sci.* 92:1670–1675

**Question From:** Dr. Derek Emmerson

**Question:** Is this condition on the radar of USDA as a potential cause for condemnation of product?

**Answer:** For the most part, no. However, I have been asked that question in the past on if the issue should result in condemnation and my answer has always been no. This is a quality issue.

**Question From:** Dr. Derek Emmerson

**Question:** Were amino acid concentrations varied in your experiment where you evaluated the influence of high energy diets on the incidence of white striping?

**Answer:** The diets for low energy and high energy treatments are below:

Table 1. Composition (% DM) of the low- (LED) and high-energy (HED) diets.

Ingredient	0 to 18 d		18 to 32 d		32 to 54 d	
	LED	HED	LED	HED	LED	HED
Yellow corn	63.638	51.217	66.254	55.579	69.351	53.914
Poultry oil	0.496	6.013	0.515	5.900	0.503	6.065
Soybean meal	32.360	39.199	30.012	35.201	27.066	36.919
Limestone	0.274	0.175	0.246	0.197	0.270	0.168
Defluorinated phosphate	1.735	1.882	1.528	1.679	1.360	1.455
Feed grade salt	0.244	0.227	0.269	0.252	0.290	0.277
MHA-84%	0.290	0.349	0.261	0.303	0.237	0.318
L-Threonine	0.060	0.063	0.041	0.041	0.041	0.041
L-Lysine HCl	0.203	0.175	0.174	0.148	0.182	0.143
Waldroup vitamins <sup>1</sup>	0.500	0.500	0.500	0.500	0.500	0.500
Mintrex P_Se <sup>2</sup>	0.100	0.100	0.100	0.100	0.100	0.100
Coban 90	0.050	0.050	0.050	0.050	0.050	0.050
BMD-50	0.050	0.050	0.050	0.050	0.050	0.050
<b>TOTAL</b>	<b>100.000</b>	<b>100.000</b>	<b>100.000</b>	<b>100.000</b>	<b>100.000</b>	<b>100.000</b>
<b>Nutrient content</b>						
Crude protein, %	21.53	23.91	20.53	22.24	19.31	22.96
Calcium %	0.90	0.95	0.81	0.86	0.74	0.79
Nonphytate P, %	0.44	0.47	0.40	0.43	0.036	0.39
Methionine %	0.61	0.68	0.57	0.62	0.54	0.64
TSAA %	0.94	1.05	0.90	0.97	0.85	0.99
Lysine %	1.27	1.42	1.18	1.29	1.11	1.33
ME kcal/kg	3002.95	3205.71	3025.00	3250.00	3063.54	3250.90

<sup>1</sup>Provided 7,715 IU of vitamin A (from vitamin A acetate); 5,511 IU of cholecalciferol; 16.53 IU of vitamin E (from dl-alpha-tocopheryl acetate); 0.013 mg of vitamin B<sub>12</sub>; 6.6 mg of riboflavin; 39 mg of niacin; 10 mg of pantothenic acid; 1.5 mg of menadione (from menadionedimethylpyrimidinol); 0.9 mg of folic acid; 1,000 mg of choline; 1.54 mg of thiamin (from thiamin mononitrate); 2.76 mg of pyridoxine (from pyridoxine HCl); 0.066 mg of d-biotin; and 125 mg methoxyquin per kg of diet.

<sup>2</sup>Provided 40 mg of Mn (as manganese methionine hydroxy analogue complex); 40 mg of Zn (as zinc methionine hydroxy analogue complex); 20 mg of Cu (as copper methionine hydroxy analogue complex); and 0.3 mg of Se (as selenium yeast) per kg of diet (Novus International, Inc., St. Louis MO).

From: Kuttappan, V.A., V. B. Brewer, P. W. Waldroup, and C. M. Owens. 2012. Influence of growth rate on the occurrence of white striping in broiler breast fillets. *Poult. Sci.* 91:2677-2685

## **Growth hormone transgenic Atlantic salmon: Opportunities, risks, and risk management**

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A large international effort has developed growth hormone-transgenic fishes of 18 species for potential use in aquaculture (Hallerman et al. 2007). The leading candidate for commercial production is the AquaAdvantage salmon, an Atlantic salmon expressing an introduced copy of the Chinook salmon growth hormone (GH) gene (Du et al. 1992). It exhibits 4-6-fold growth rate enhancement early in life (Fletcher et al. 2004) and a 10–20% improvement in feed conversion efficiency (Entiss 1997, Cook et al. 2000a), offering the prospect of shorter production time, reduced costs, and improved efficiency and profitability. AquaBounty Technologies seeks U.S. Food and Drug Administration approval for limited production of the fish under strict confinement in Panama (FDA 2010a); full-scale commercial production is not sought at this time. Key issues pertain to food safety and environmental risks associated with its production (NRC 2002).

Consumers concerns regarding genetically modified foods are prominent, including concerns regarding food products from transgenic animals. The level of food safety concern associated with a GM animal product varies with the application (NRC 2002). Focusing on the AquaBounty salmon, bioactivity of the transgene does not pose food safety concern; that is, neither salmon growth hormone, not its fragments, nor hormones secreted in response to growth hormone pose risk to consumers. Regarding allergenicity, the growth hormone molecule is a small peptide, present at low titer, and has no known allergenic epitopes; further, consumers allergic to fish would not buy this fish product. Against this background, the Food and Drug Administration Veterinary Medicine Advisory Committee concluded that products from the AquaAdvantage Atlantic salmon posed no food safety issues (FDA 2010a). Despite the lack of science-based food safety issues, many consumers want to know whether foods are GM and make informed product choices. In the European Union and Japan, labeling of GM-derived food products is required. In the United States, however, such labeling is not required and is the subject of ongoing debate (FDA 2010b).

The issue of ecological risk posed by production of transgenic fishes has a recognized scientific basis (NRC 2002, Kapuscinski et al. 2007). The classical framework for assessing ecological risk (NRC 2002) has five steps. First, risk assessors identify potential harms, i.e., ecological or genetic outcomes that we would not want to become realized. Second, they identify the hazard that might lead to those harms, in this case the transgenic fish. Third, they assess the probability of exposure, in this context the likelihood of escape and persistence of transgenics in a particular receiving ecosystem. Fourth, in the most critical step, they assess the probability of harm becoming realized given exposure to the hazard. Finally, ecological risk is calculated as the probability of exposure to the hazard times the probability of harm being realized given exposure.

Empirical studies of the growth hormone-transgenic Atlantic salmon have shown that transgene expression affects not only growth rate, but also a range of morphological, physiological, and behavioral traits. These studies have shown mostly maladaptive alterations of oxygen and energy metabolism (Stevens et al. 1998, Stevens and Sutterlin 1999, Cook et al. 2000b, 2000c), feeding behavior (Abrahams and Sutterlin 1999), smoltification (Saunders et al. 1998), and cardiorespiratory function (Deitsch et al. 2006). These negative impacts have led some observers to suggest that the transgenics would pose no significant environmental risk. However, empirical observations of GH-transgenic fishes also have shown heightened growth rate, heightened food conversion efficiency, in some cases larger ultimate size (which may confer mating advantage), and increased osmoregulatory ability. Noting that trait-by-trait assessments of fitness do not address the integrated phenotype of an individual, especially if there are tradeoffs among fitness-related traits, how should we predict the fate of the transgene in receiving populations and hence, likelihood of harm? The answer is that we should consider the net effect of transgene expression on the fitness of individuals (Muir and Howard 1999, 2001, 2002). Against this background, I was the principal investigator for a recent collaborative project involving Ewen McLean (then at Virginia Tech) and Ian Fleming and Garth Fletcher (Memorial University of Newfoundland). Our goal was to develop empirical data useful for quantifying ecological and genetic risks posed by GH transgenic Atlantic salmon in the wild.

The first broad question that we asked was whether heightened competition, predation, or other processes pose ecological harms to receiving ecosystems. Focusing upon the survival component of fitness, we found no difference between transgenics and controls regarding oxygen consumption rate, developmental rate, survival until emergence from gravel, fry behavior, or growth and survival in an artificial stream (Moreau et al. 2011a). In a second set of experiments, we asked whether transgenics are compromised in the face of environmental stress. We applied ecologically and aquaculturally relevant stressors – starvation, low Dissolved oxygen, and handling – and followed stress response variables including hematocrit, pH, pCO<sub>2</sub>, Ca<sup>++</sup>, K<sup>+</sup>, Na<sup>+</sup>, Cl<sup>-</sup>, glucose, and cortisol. Cnaani et al. (in review) found that transgenic fish were more stressed than wild-type fish, showing faster and more pronounced stress responses. Overall, these data sets indicate that the GH-transgenic Atlantic salmon are equally or less fit than wild type salmon; yet, under a range of ecological conditions, fitness could be sufficiently high that there could be considerable risk of ecological harm becoming realized.

The second broad question that we asked was whether interbreeding of transgenic fish with wild populations poses genetic and evolutionary harms to receiving populations; that is we sought to quantify the reproductive component of fitness. We should note that introgression of the transgene into a wild population is a risk pathway, but not a risk endpoint, i.e., it is not a harm in and of itself. Possible harms would include loss of adaptation, reduced genetically effective population size, and in the extreme case, extinction of receiving population. Noting that rapid growth rate is related to early maturation in salmon, we predicted that precocious maturation of the salmon as parr may pose a potent route for introgression of GH transgene. However, transgenics matured as parr *less* frequently than non-transgenics (Moreau and Fleming 2012), and so the opportunity to introgress is likely *reduced* for transgenic parr. In our most critical experiment, we compared the behavior and reproductive success of transgenic and control males in artificial spawning channels. We had trials where transgenic and non-transgenic anadromous males competed for breeding opportunities and other trials where a transgenic or non-transgenic anadromous male had sole access to female. We found that transgenic parr were inferior competitors relative to wild-type parr in terms of nest fidelity, spawn participation, and fertilization success (Moreau et al. 2011b). We found that transgenic

anadromous males were outcompeted in terms of nest fidelity, quivering frequency, and spawn participation. Tying together the results of both spawning experiments, transgenic males exhibiting either reproductive strategy exhibited low but non-zero reproductive fitness. Hence, the risk associated with reproduction of GH-transgenic Atlantic salmon may generally be low, but it is non-zero.

Pulling together all our results to predict the net fitness of GH-transgenic Atlantic salmon and transgene fate in near-natural ecosystems, we found that the survival component of fitness was less than or equal to that of the wild type, and reproductive fitness decreased relative to the wild type. The upshot is that net fitness is reduced, and the transgene would be expected to be purged from a receiving population following a single episode of introduction. We did not, however, address the outcome should introductions recur via continuing escapes from aquaculture operations.

The adoption of risk management measures affects the assessment of associated risk; that is, ecological risk may be minimized by culturing transgenic fish under strict confinement. Hence, the AquaBounty proposal before the U.S. Food and Drug Administration is for pilot-scale production of GH-transgenic Atlantic salmon in Panama, in indoor recirculating aquaculture systems with redundant physical confinements and reproductive confinement (FDA 2010a). This production experiment is aimed at evaluating production economics and demonstrating effective confinement. Should the outcomes prove positive, AquaBounty would have to seek further regulatory approval for any expanded production.

It becomes clear that regulatory approval is key to the future of the AquAdvantage salmon. To date, no genetically modified animal intended for use as food by humans has received regulatory approval. However, on December 26, 2012, the U.S. Food and Drug Administration released its draft environmental assessment (FDA 2012a) and its preliminary finding of no significant impact (FDA 2012b) for the proposed pilot-scale experiment for production of the GH-transgenic Atlantic salmon. The public comment period was open through April 26, 2013. Whatever the regulatory outcome, the FDA decision on the AquaBounty salmon will be critical to the commercialization of transgenic fishes and

transgenic animals more generally. There are other transgenic fishes in the R&D and regulatory pipeline, including other GH-transgenic fishes, disease resistant lines, biopharm lines, and reversibly sterile lines. Other transgenic animals in development include goats expressing lysozyme, which reduces milk bacterial loads. The “Enviropig” expressed phytase, conferring better utilization and lower excretion of dietary phosphorous, but recently it was euthanized because of ongoing cost and regulatory uncertainty. Porcine models of disease are hung up in regulatory review because they could potentially be eaten by humans (Maxmen 2012). A recent review concluded that regulatory uncertainty is stifling development of genetically modified animals (Van Eenennaam et al. 2011)

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**Questions for Speaker:**      **Dr. Eric Hallerman**

**Question From:**      **Dr. Karim Akbar**

**Question:** What is the mechanics of measuring feed efficiency in salmon or other experimental fish? You mentioned that feed efficiency in fish is good. If the test is done in tank with fish having no access to other sources of feed, that is fine, but some reports are based on experiments in open net enclosures, and only feed that is added is considered.

What about sources of feed that are already in the water and are not considered in the calculation?

**Answer:** Measurement of feed efficiency in fish generally is quantified as mass of feed provided per unit of mass gained, e.g., 1.6 grams of feed per gram gained. Dr. Akbar is on target, some past studies have reported excellent feed conversion ratios that actually were unreliable because fish held in outdoor systems had access to natural food items. Yet, fish really do have excellent feed conversion efficiency. Lovell (1989) compared feed conversion ratios of 1.2 for channel catfish to 2.1 for broiler chickens and 7.7 for beef cattle. Why is it, then, that fish have such good feed conversion ratios? First, they are cold-blooded and allocate no energy to maintaining body temperature. Lovell notes that fish are able to assimilate diets that are high in protein because of their lower dietary energy requirement. Second, living in water, in which they are neutrally buoyant, fish allocate little energy and material resource to development of their skeleton. Third, fish convert food that is 90% dry matter to fish flesh that is ~15% dry matter. Hence, while feed conversion efficiency in fish is excellent, it is not miraculous.

**Question From:** Dr. Casey Owens Hanning

**Question:** Are there any meat quality differences due to transgenic Atlantic salmon?

**Answer:** Many aspects of meat quality of the AquaBounty Atlantic salmon were quantified in the context of the company's application to the Food and Drug Administration for approval of their proposed pilot-scale production trial. Results were presented at a public meeting held in 2010 (FDA 2010). Of the proximate, vitamin, mineral and amino acid analytes quantified, only three – vitamin B6, folic acid, and niacin – were present at levels in transgenic salmon that were statistically significantly different from those in control salmon. Based on all criteria considered, the FDA Veterinary Medicine Advisory Committee concluded that the levels of all proximate, vitamin, mineral and amino acid analytes in transgenic salmon except vitamin B6 were similar to levels in one or more appropriate groups of control salmon. They did note, however, that sample sizes in some cases were not particularly large.

**Question From:** Dr. Jesus Arango

**Question:** Public perception is an important driver in product demand. Fish and salmon meat in particular, have seen an explosion in demand mainly due to the perception of being healthy. However, there are several sources that warn about the presence of heavy metals in fish meat. What is your opinion? Would it vary depending on sources? Should one be really concerned about this issue?

**Answer:** Heavy metals tend to bioaccumulate up marine food chains, such that high-level predators such as tunas and billfishes can carry high levels of mercury relative to their environment. Consumption advisories have been issued for certain such species, typically recommending no more than one serving per month.

The most prominent issue pertaining to cultured fishes, however, pertains to organic compounds. Hites et al. (2004) analyzed farmed and wild salmon products for

organochlorine contaminants and showed that these contaminants (e.g., PCBs, DDT, and dioxins) were significantly higher in farmed salmon than in the wild. Interestingly, European-raised salmon had significantly higher contaminant loads than those raised in North and South America. Risk analysis indicated that consumption of farmed Atlantic salmon may pose health risks that detract from the beneficial effects of fish consumption. The Hite et al. report led to an active exchange of letters and responses; see *Science* for 23 July 2004. My recommendation is to enjoy salmon in moderation as part of a balanced diet.

**Question From:**        **Damarius Fleming**

**Question:** In the face of resistance to GM foods by commercial markets (groceries, etc.) do you feel that researchers are doing enough to dispel some of the rumors/trepidation of consumers?

**Answer:** Many consumers perceive risks from consumption of genetically modified foods. To the extent that these perceptions stem from lack of information about GM foods, they can be addressed by access to science-based information. It is incumbent upon researchers in the academic and agribusiness communities to develop appropriately targeted information packages and present them for consideration by the general public in appropriate fora. For example, Van Eenennaam et al. (2011) explained the science and regulation of food from genetically engineered animals, engaging the public through the Council for Agricultural Science and Technology. The Cooperative Extension Service in every state engages a wide cross-section of Americans, and is a useful outlet for effectively communicated scientific information. Public acceptance of GM foods ultimately determines the success or failure of such products.

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# What do RFI, PRRS, and SCID have in Common?

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## Introduction

RFI refers to Residual Feed Intake, a measure of feed efficiency defined as the difference between an animal's observed feed intake and its expected feed intake based on expected energy requirements for maintenance and production, based on observed performance (e.g. body weight, growth rate, fatness). It has been used as a measure of feed efficiency in most livestock species, including chickens and pigs.

PRRS stands for Porcine Reproductive and Respiratory Syndrome. Caused by an RNA virus, it is the most costly disease in the pig industry, both in the US and globally. Annual costs to the US industry alone are estimated at 664 million (Holtkamp et al. 2013). PRRS affects both the breeding (piglet production) and the growing pig sectors of the pork industry through a decrease in reproductive health, an increase in deaths, and reductions in the rate and efficiency of growth. PRRS also functions as a cofactor in other disease syndromes. Efforts to control the disease through improvements in veterinary health management, biosecurity, disease elimination, and vaccination have been met with some success but not enough to stem the spread of the disease. Host genetics is another route that is currently being pursued.

SCID stands for Severe Combined Immune Deficiency. It is a category of devastating genetic diseases that results in lack of or severe impairment of the adaptive immune system. It can be caused by a mutation in genes that are crucial to the development of antibodies. It has been identified in dogs, horses and humans (David Vetter, the Bubble boy) and has been artificially created in mice, rats, and recently pigs (Suzuki et al. 2012). SCID mice and rats are extensively used as models for biomedical research into cancer, vaccine development, etc.

So what do RFI, PRRS, and SCID have in common, apart from the fact that all three are acronyms? The purpose of this paper is to tell the story of how the paths of these three rather distinct phenomena crossed in research involving our selection lines for RFI in pigs at Iowa State University, thereby providing insight into the sometimes serendipitous route that

research can take and into findings that may also be of relevance to the poultry breeding industry.

### **The ISU RFI lines in Yorkshire Pigs**

In order to enable study of the genetic and physiological basis of feed efficiency in pigs, a selection experiment in Yorkshire pigs was started at Iowa State University in 2001, with the aim to develop lines that differ in RFI during the grow-finish phase (Cai et al., 2008). Using purebred Yorkshire pigs sourced from the Midwest, a selection line for low RFI (= increased efficiency) and a randomly selected control line were initiated by splitting litters. In generation 5, selection for increased RFI (= reduced efficiency) was initiated in the randomly selected control line. Now in its 8<sup>th</sup> generation of selection, the low RFI line consumes 376 g/d less than the high RFI line and has 241 g/d lower RFI (Figure 1). Selection for RFI has resulted in an improvement in feed conversion ratio, with the low RFI line requiring 0.22 g feed less per g weight gain than the high RFI line in generation 8. Other performance traits that have changed as a result of selection for RFI are average daily gain and ultrasonic backfat and loin eye area, with low RFI pigs growing 79 g/d slower, having 2.5 mm less backfat, and 1.5 cm<sup>2</sup> larger loins than pigs from the high RFI in generation 8.

In recent years, the focus of research using the ISU RFI lines has shifted towards understanding the biological and physiological basis of the differences in feed efficiency that have been established through selection between the lines and towards identifying bio- or genetic markers that are associated with feed efficiency (Young and Dekkers, 2012). In addition, research has been initiated to determine how the two lines respond to different stressors and challenges, as part of a large USDA-NIFA grant (grant # 2011-68004-30336).

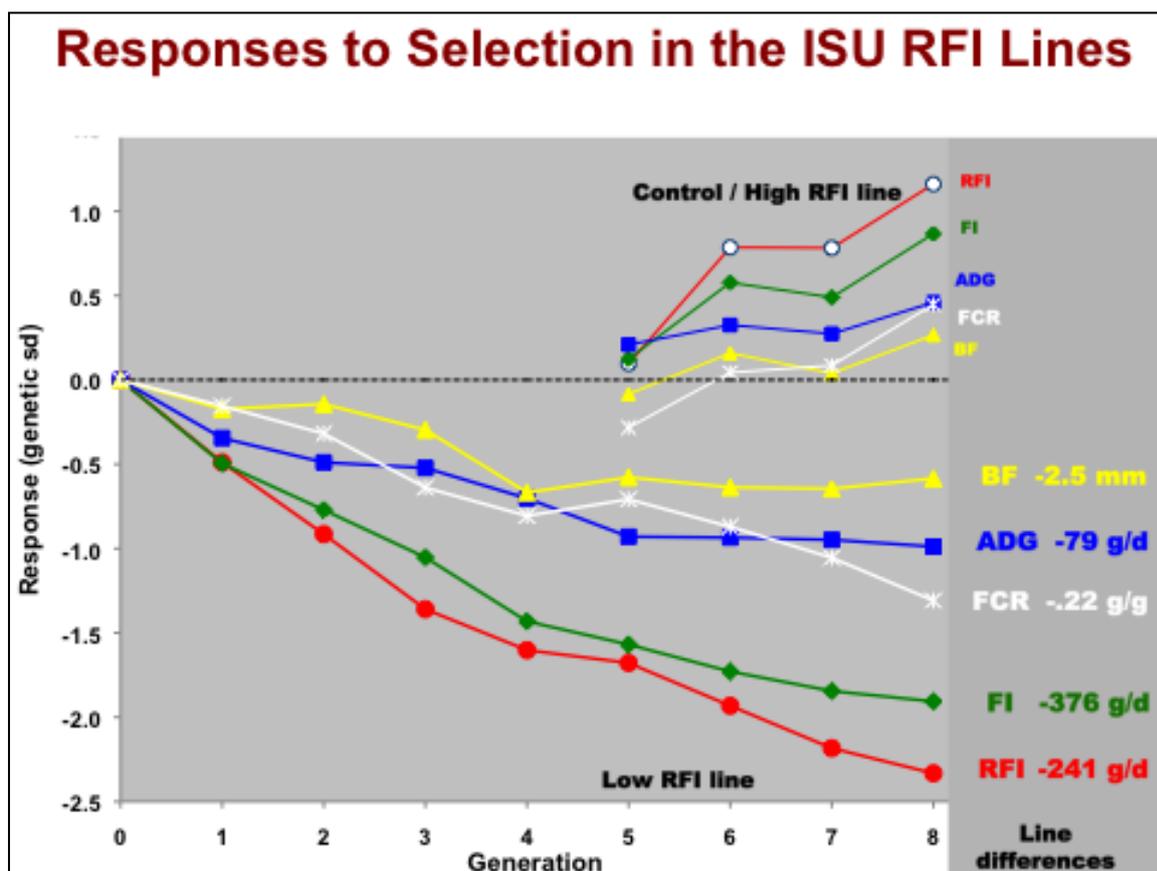
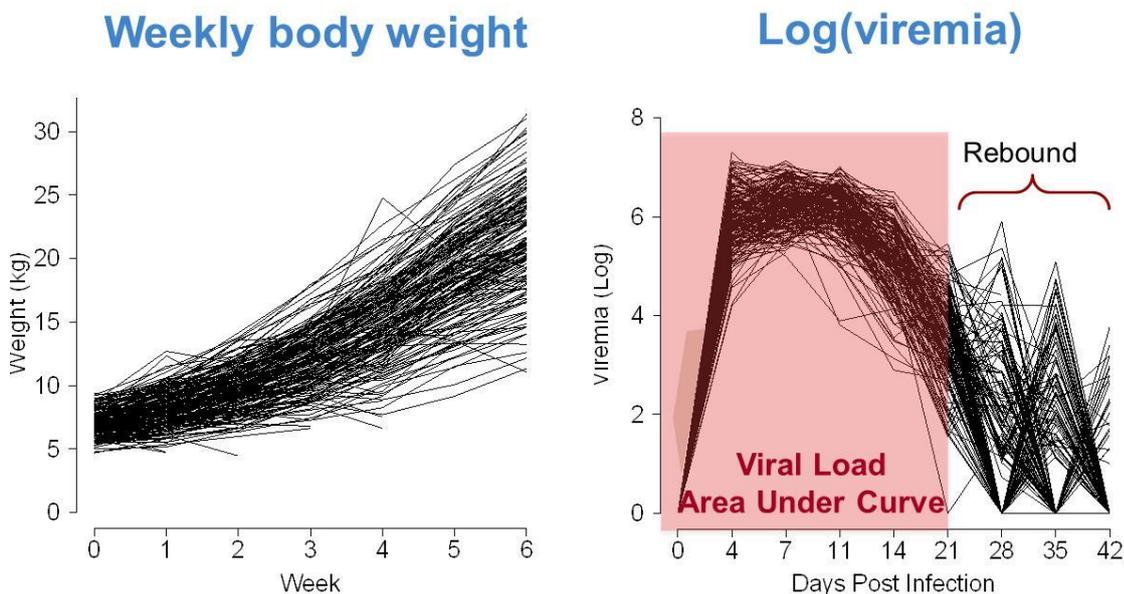


Figure 1. Responses in the ISU RFI selection lines (from Young and Dekkers, 2012).

### PRRS Host Genetics Consortium

The PRRS Host Genetics Consortium (PHGC) was initiated in 2007 with funding from the National Pork Board, USDA-NIFA and industry breeding organizations (Fast Genetics, Genesis, Genetiporc, Newsham, PIC, Topigs) (Rowland et al. 2012). The objective of the PHGC is to use the modern tools of genomics to identify genes/genomic regions that are associated with increased resistance or reduced susceptibility of piglets to PRRS virus infection. The PHGC uses a nursery pig challenge model, in which groups of 200 commercial crossbred pigs, provided by the breeding companies, are brought to a challenge facility at Kansas State University, shortly after weaning. Following a 1-week acclimation period, piglets are infected with a specific strain of the PRRS virus and followed for 42 days, with frequent weighing and blood collection. Figure 2 shows typical results that are obtained from a group of 200 piglets, showing large variability in growth following challenge, and in the rate of recovery from infection in terms of viremia levels in serum. A notable finding was

the ‘rebound’ in viremia that occurred in about 30% of the pigs after they had started to clear the virus. This rebound was, however, found not to be heritable and likely is more the result of changes in the virus populations than the host. Thus, our analyses have focused on levels of viremia prior to 21 days post infection, quantified by area under the curve, as illustrated in Figure 2, which we refer to as viral load. Viral load was found to have a substantial heritability of 0.41 ( $\pm 0.13$ ) in these experimental challenges, while growth during 42 days post infection was moderately heritable at 0.29 ( $\pm 0.11$ ). The estimate of the genetic correlation between viral load and weight gain was -0.47, but with a high standard error of 0.22. These estimates were based on data on about 1,500 pigs from 5 genetic sources, representing crosses between four breeds (Duroc, Landrace, Large White, Pietrain).



**Figure 2.** Example results for body weight and serum viremia in a group of ~200 piglets following challenge with the PRRS virus.

To identify genes or genomic regions associated with host response to PRRS infection, all piglets were genotyped with the Illumina Porcine SNP60 Beadchip and a genome-wide association study (GWAS) was conducted. Results showed a strong effect on chromosome 4 that explained about 15% of the genetic variance for viral load and about 11% of the genetic variance for weight gain. Initial results of this finding were described in Boddicker et al. (2012), using data from the first 3 trials, which included piglets from the same genetic cross and source, but these analyses have since then been extended to data from 8 trials. A remarkable finding is that the chromosome 4 effect was present in each trial analyzed,

representing different breed crosses and genetic sources. Although the exact gene and mutation have not yet been identified, the favorable alleles for the region are present in all breeds and lines analyzed but at a low frequency, which offers great perspectives for genetic improvement through marker-assisted selection on this region.

### **PRRS and the ISU RFI lines**

In order to evaluate whether the ISU RFI lines responded differently to a PRRS challenge, 100 piglets from each line were sent to Kansas State University, where they were challenged with the PRRS virus, following the PHGC protocol. Our hypothesis was that, having been selected under relatively high-health conditions, the low RFI line (high efficiency) would not have the resources to mount as effective an immune response to the PRRS virus as the high RFI line. However, our hypothesis was proven wrong since, although differences were not significant at  $p < 0.05$ , the low RFI line tended to have a lower viral load ( $p = 0.09$ ) and a higher growth rate following challenge ( $p = 0.17$ ). Mortality was less than 10%, similar to that observed in other PHGC trials, and was not different between the two lines. This finding is consistent with other results from the two lines, that suggest that the efficient line is better able to mobilize resources and direct it where they are needed to deal with a challenge or demand. For example, the low RFI line has slightly greater litter size, both at birth and at weaning, and low RFI piglets are slightly heavier, both at birth and at weaning. The low RFI sow is able to provide the required extra nutrients to its piglets not by eating more but by mobilizing more body reserves (Young et al. 2010).

### **SCID, PRRS, and the ISU RFI lines**

One of the routine procedures in the Kansas State University PRRS challenge studies is to conduct a necropsy on pigs that died or that had to be euthanized prior to the end of the experiment, in order to determine the cause of death. Routine necropsy of 4 of the piglets from the low RFI line showed some interesting findings: a very small thymus and lymph nodes and other abnormal histopathology, and the piglets had no detectable antibodies to the PRRS virus. These findings were recognized as being consistent with SCID, resulting in the first reported cases of SCID in pigs (Cino Ozuna et al. 2012). Remating the six parents of these four piglets resulted in litters that each produced SCID piglets in an approximately 1:3 ratio, indicating that it segregates in an autosomal recessive manner.

At birth, SCID piglets appear healthy without a weight difference between affected and normal littermates. Pre-suckling umbilical cord blood of SCID piglets were shown to have no detectable immunoglobulins. However, suckling provides affected piglets with maternal antibodies, which allow SCID piglets to remain healthy for several weeks after farrowing. But maternal antibody levels diminish after weaning, leaving SCID piglets susceptible to many pathogens and they will succumb to these, unless they are maintained in a pathogen-free environment, similar to the Bubble Boy. SCID piglets have been shown to have no or very low levels of B and T cells, which are needed to mount an adaptive immune response. The SCID piglets do have natural killer cells but it is not clear whether they are functional in the SCID pig.

To identify the gene or genomic region responsible for SCID, the Illumina Porcine SNP60 Beadchip was used to genotype 20 affected piglets, 6 known carrier parents, 50 unaffected littermates, and 97 ancestors. Using GWAS, a 5.6 Mb region that likely contains the causative mutation was identified. This region contains a strong candidate gene based on interspecies comparative information. Phasing the SNPs in a 1 Mb region surrounding the candidate gene showed two haplotypes that segregate with affected status, either in a homozygous or compound heterozygous state. The identified haplotypes were traced through the pedigree of the RFI line back to the founder generation, which were sourced from the purebred Yorkshire population. Allele-specific primers were developed to allow identification of carriers and affected pigs in the ISU RFI population. Work is ongoing to identify the causative mutation.

### **SCID Pigs as a Biomedical Model.**

Transgenic SCID mice are extensively used as a biomedical model for immune system research, cancer research, studies of the effects of disease, cell and tissue transplantation, and testing new vaccines and therapeutic agents for immuno-compromised individuals. However, it has been well established that pigs are much better biomedical models for humans than mice. Until our finding, and the recent production of a transgenic X-linked SCID pig in Japan (Suzuki et al., 2012), no such pig models were available. The transgenic SCID pig developed by Suzuki et al. (2012) is X-linked and produces a different immunophenotype than our SCID pig.

To demonstrate the utility of our SCID pig as a biomedical model, 3 SCID piglets and 3 normal littermates were sent to Kansas State University, where human melanoma and human pancreatic carcinoma cells were injected in the left and right ears of the piglets (Basel et al., 2012). While the normal piglets immediately rejected and cleared the human cells, in the SCID piglets, these human cells formed visible tumors. This confirms that the SCID piglets do not have the ability to reject foreign cells and demonstrates that they provide a useful model for cancer and other research.

Because SCID carriers are phenotypically normal, SCID piglets can be produced by carrier by carrier matings. However, this only produces 25% affected piglets on average, which does not make a very reliable and efficient system to produce SCID piglets for research. In humans, as well as other species, the standard procedure for curing SCID is by giving the patient an adaptive immune system through bone marrow transfer. Thus, in order to rescue affected SCID piglets such that they can be raised to breeding age and be available for breeding as SCID homozygotes, nine affected piglets from four litters were given bone marrow transfers from normal donors that were matched based on major class I and II haplotypes of the MHC. Five recipients showed signs of graft versus host disease after transfer and were euthanized. The four surviving transplanted piglets were documented to have a reconstituted adaptive immune system and are providing a pool of frozen semen for use to breed carrier females to produce litters with 50/50 SCID/normal piglets.

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**Questions for Speaker:**      **Dr. Jack Dekkers**

**Question From:**      **Dr. Neil O’Sullivan**

**Question:** Signal found on SSC4 for growth rate. Does this signal show up in tests done on a normal clean farm?

**Answer:** We have not been able to find reports of QTL for this region in the literature. Most/all of the reported QTL studies would have been conducted without presence of PRRS and in relatively ‘clean’ environment. So this suggests that this region may not affect performance in a ‘clean’ environment.

**Question From:** Dr. Sue Lamont

**Question:** Following up on Rowland's hypothesis about macrophage permissiveness and PRRRs, what are the percentages (or absolute numbers) of macrophages present in SCID vs. normal pigs?

**Answer:** We don't know. To date, all our flow cytometry data to investigate cell numbers are based on blood and we have not specifically looked at macrophages. Macrophages only comprise a very small portion of the cells in blood.

**Question From:** Dr. Sue Lamont

**Question:** In the grafted pigs, from where did the host-genotype WBC come, which were detected in the early days post-graft?

**Answer:** We have not determined their origin but they likely are of donor origin. We have determined that our SCID pigs are deficient in B and T cells but not in natural killer cells. In addition to representing cells other than B and T cells, they could represent lymphocytes derived from colostrum as there is some evidence that lymphocytes can cross the intestinal barrier in newborn piglets. Finally, we have not determined whether our SCID pigs are complete devoid of B and T cells; there could be some 'leakiness', which is often observed in SCID models, and/or the B and T cells that are there may not be functional.

**Question From:** Dr. Gerald Herbert

**Question:** Were the high heritability figures presented a function of the animals (the initial populations) being crossbred?

**Answer:** That could play a role but within a trial, all piglets were of the same cross and trial was included as a fixed effect in the model of analysis, so it is unlikely that breed differences contributed to the estimate of heritability.