AN ABSTRACT OF THE THESIS OF

Michelle A. Charbonneau for the degree of Master of Science in Poultry Science presented on January 17, 2007

Title: The Influence of an Elevated Incubation Temperature on the Expression of Clubbed Down in Coturnix Embryos

Abstract approved: __________________________________________________

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Hatchability of the fertilized avian egg is impacted by factors which can be categorized as being either genetic or environmental in nature. An elevated incubation temperature, > 100 F reduces development time (time, days to hatch), but increases embryonic mortality and lowers the numbers of fertile eggs that will hatch.

Embryonic failures, which affect hatchability are expressed at varying times in development. One such embryonic period is associated with down formation. In the mid-shaft portion of the developing feather plumule a condition referred to as clubbed down has been described. This condition was recognized in 1937, but the precise origins of this disorder remain unclear. Genetic studies conducted in this thesis have revealed that clubbed down in Coturnix quail is the expression of an autosomal recessive gene in the homozygous state whose expression is modulated by incubation at a temperature of 102 F. Results of selected matings of carriers for
clubbed down suggest that the condition is expressed in embryos that are homozygous for the recessive gene. The responsible gene appears to be temperature sensitive in its expression.
The Influence of an Elevated Incubation Temperature on the Expression of Clubbed Down in Coturnix Embryos

by
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The Influence of an Elevated Incubation Temperature on the Expression of Clubbed Down in Coturnix Embryos

CHAPTER 1

INTRODUCTION

On March 1, 2005, it was reported in the US that there were 659 million broiler-type and 34.7 million egg-type eggs in incubators, for a total of 693.7 million eggs (NASS, 2005). These numbers provide an estimate of the economics associated with hatching chicken eggs in the U.S. Since not all eggs incubated will hatch, if improvements in hatchability can be achieved through enhancing our understanding of avian genetics and incubation requirements, a significant economic impact would be realized by the poultry industry.

Hatchability can be influenced by many factors which can be categorized as either genetic or environmental in nature. Of particular interest is the interaction of genetics and the environment. Is there a way to manipulate an environmental factor, such as incubation temperature, so that genes can be altered resulting in an increase or decrease in the appearance of lethal genetic conditions? An elevated incubation temperature shortens development time (hours to hatch), but increases embryo mortality, while a lower incubation temperature extends development time and also increases mortality. If an increase in incubation temperature
shortens the time to hatch, is it economically feasible to lose birds due to increased mortality?

Genetic embryonic failures which effect hatchability, can occur at any age during embryogenesis. Some failures occur, at the onset of the circulatory system’s formation, during limb differentiation, and others during down formation, etc.

There are three physical regions of the down plumule where abnormalities have been observed and subsequently described. At the distal end, a condition known as knobby has been studied in the turkey embryo (Smith and Smyth, 1970). At the proximal region of the feather plumule a condition referred to as swollen down was also described in turkey embryos (Savage et al., 1986). In the mid-shaft portion of the feather plumule, a third condition known as clubbed down has been observed. This condition known as clubbed down was first described in 1938 by Lepkovsky and collaborators. The precise origins of this disorder still remain unclear.

Clubbed down is a cause of embryonic mortality in developing avian embryos. Previously, it has been believed that clubbed down was due to a riboflavin deficiency in the embryo (Lepkovsky et al, 1938; McClymont and Hart, 1947; Ambrose, 2002). There has been some uncertainty about the riboflavin deficiency explanation. This uncertainty is due to a belief that the condition may have a
heritable basis for its origin (Hawes and Buss, 1965). Furthermore, the probable genes associated with clubbed down formation may be affected by incubation temperature (Colvin, 2005). In a recent long-term study involving the genetic selection of Coturnix embryos to accelerate embryonic rate of development at an elevated incubation temperature (102 F) embryos with clubbed down expression were observed (Colvin, 2005). The incidence of clubbed down appearance varied among egg settings suggesting that the clubbed down condition may have an environmental origin, however its incidence was restricted to selected paired matings suggesting a heritable origin. Two questions arise from this information, the first, could genetics be responsible for the presence of clubbed down in embryos that developed at 102 F? The second question, if there is a heritable basis for clubbed down, how or does incubation temperature affect the gene/s that cause clubbed down? These questions provide the basis for the following thesis.
CHAPTER 2

LITERATURE REVIEW

Feather Function

There are three main types of feathers: the contour feather, the down feather and the filoplume (Hodges, 1974). The predominant feathers on a bird’s body are the contour which are specialized for flight. Contour feathers are located on the bird’s outer body as well as the wings and tail. Down feathers, whose primary function appears to be for insulation, comprise the under plumage of a bird and are usually concealed beneath the contour feathers. Filoplumes are always situated beside other feathers. They are simple, hair like structures that grow in circles around the base of contour or down feathers. Filoplumes are used for aerodynamics by aiding in the adjustment of contour feathers during flight (Lucas and Stettenheim, 1972).

Feather Structure

Contour feathers contain a central shaft that can be divided into two parts. The first part is the bare calamus, which lies partly within the follicle. Moving up from the calamus is the second part of the feather shaft known as the rhachis, which
carries supports the vane. Barbs are joined together by barbules which project from either side of the rhachis (Hodges, 1974).

**Down Formation**

The formation of chick embryonic down begins with the appearance of feather primordia at 7 days of incubation with the condensation of mesenchymal cells into a layer of dermis. This dermis forms into a line within each presumptive feather tract. The first visible signs of feathers are clusters of epidermal cells described as placodes. At the same time the placodes form, dermal cells congregate and form clusters beneath the placodes. The epidermal placode and the dermal condensation beneath it comprise the germ, which is the rudimentary portion of a feather (Lucas and Stettenheim, 1972). Each line of dense dermis and overlying epidermis dissociates into a row of feather primordia. The feather primordia soon assume the appearance of tiny humps on the surface. The patterns of feather tracts are positioned in such a way that a new row of primordia forms on each side of the first as the cells congregate opposite the spaces between the original humps. Feather primordia are distributed in a hexagonal pattern so that each feather is surrounded by six other feathers. This pattern continues until virtually all follicles on the embryo are formed (Lucas and Stettenheim, 1972).
The dermis is believed to induce the epidermis to begin its differentiation during early feather germ formation. During this time, details of feather structure and the precise order of feather growth within a tract are thought to be determined. The dermis then loses its inductive capacity and the epidermis assumes control. The epidermis then induces the dermis to form a core of vascularized pulp and the epidermis determines the specific properties of the feathers. The dermis retains only the specific capacity to stimulate the epidermis to produce a feather (Lucas and Stettenheim, 1972).

At 7 days of incubation the earliest feather germs appear on the embryo’s back. At this time, basal epidermal cells of a placode continue to elongate as the dermis is condensing beneath. Epidermal cells often appear to aggregate in stratified layers toward the center of each placode. A feather germ begins to emerge shortly after it has reached the preceding stage. This elevation occurs at about 7 ½ to 8 days for the first formed germs on the back (Lucas and Stettenheim, 1972).

Further differentiation of the feather germ occurs between 9 and 16 days of incubation. The intermediate layer of the epidermis is nearly responsible for the derivation of all of the parts of a feather. The young feather germ pushes above the surface of the embryo’s body because of rapid cell proliferation. The feather germ can be described as a ‘finger’ of dermis covered by a thin layer of
epidermis. In the chicken, feather germs are microscopically visible by day 9 of incubation (Lucas and Stettenheim, 1972).

Towards the end of the 10\textsuperscript{th} day of incubation, growth of the feather germ accelerates rapidly. The cells of the intermediate epidermis rearrange into a series of ridges that are parallel to the long axis of the feather germ. These ridges are known as barb ridges because they are rudiments of the barbs (Lucas and Stettenheim, 1972).

On the 11\textsuperscript{th} day of incubation, the epidermis at the base of the germ begins to migrate downward into the dermis. The invagination of the germ results in the separation of the rudiments of the feather follicle from the feather itself (Lucas and Stettenheim, 1972).

By day 12 of incubation, many feather germs have sunk deeper into the dermis. The follicles have become more distinct. As invagination and outward growth continue, the follicle will form a deep narrow pit and the feather germ resembles a long cylinder emerging from of the follicle. The follicular cavity lies between the follicle wall and the feather sheath and extends nearly to the bottom of the follicle (Lucas and Stettenheim, 1972).
At the base of the feather germ, the epidermis has become thickened by cells proliferating and forms a ring known as the ‘epidermal collar’. The epidermal collar surrounds a mound of dermis, the dermal papilla. The dermal papilla produces pulp which is used by the feather as a source of nutrition during feather growth (Carlson, 2002). Proximal to the tip of the feather germ, the intermediate epidermis of each barb ridge begins to differentiate into the beginnings of the barbule plates which are the rudiments of the barbules. At the center of the feather germ is a cylinder of pulp as well as an axial artery (Lucas and Stettenheim, 1972).

During feather growth, cell division occurs predominantly at the base of the feather germ and the parts of the feather differentiate as they move upward. The distal and peripheral parts of a growing feather are more fully developed than the proximal and central portions. The tips of the barbs are formed before the bases. In feathers with a rhachis, the barbs at the distal end are formed before those of the proximal end. After the barbules have formed, they attach to the rami, and the completed barbs then attach to the rhachis. The rhachis and barbs form before the calamus (Lucas and Stettenheim, 1972).
While the rudiments of the feathers and follicles have been forming, synthesis of embryonic keratin has also occurred. On day 13 the first signs of keratin can be detected in the feather sheath near the tip of advanced feather germs.

By day 14 of incubation, the developing feathers project further above the surface and have sunk deeper into the skin. The wall of the follicles is more differentiated than before. Follicular cavities have disappeared because the feather germs completely fill the follicles. The lining of a follicle and the sheath of a feather now appear as one layer. At the mouth of the follicle, the very thin follicular lining separates from the thick follicular sheath (Lucas and Stettenheim, 1972). Inside the differentiating feather, there have now formed as many as 15 barb ridges. The components of the barb ridges are now distinct in the portion of the feather germ outside the follicle. The cells of the barbule plates are compressed and begin to fuse together end to end, forming columns that will become barbules (Lucas and Stettenheim, 1972).

By day 16, the parts of the feather at the proximal end have completed their development. The pulp of the earliest developed feather germs exhibit spaces between the epidermis and the wall of the axial artery indicating that the axial artery has begun to be resorbed. The corneus layer of the follicular epidermis near the base of the follicle is continuous with the feather sheath. The fully keratinized
sheath separates from the lining of the follicle. Within a feather rudiment, the barb ridges have each differentiated a ramus near the inner edge and two masses of barbules near the periphery (Lucas and Stettenheim, 1972).

**Down Abnormalities**

There are three down abnormalities that have been described in poultry: swollen down (Savage et al., 1986), clubbed down (Lepkovsky et al., 1938) and knobby (Smith and Smyth, 1970). All of these abnormalities are characterized as a regionalized swelling of the down plumules: proximal, mid-shaft and distal, respectively. In the proximal region of the shaft, swollen down was described in the turkey (Savage et al., 1986) Swollen down is an embryonic lethal condition characterized by a swelling of the dermal pulp cavity of down feathers. Embryonic lethality ranges from about 20 days of incubation through the pipping stage (Savage et al., 1986). In the mid-shaft region of the feather shaft, the swelling known as clubbed down has been observed in chickens, turkeys and ducks (Lepkovsky et al., 1938; Hawes and Buss, 1963; Hawes, 1965). Located at the distal end of the plumule is knobby which has only been observed in the turkey (Smith and Smyth, 1970). Knobby is a semi-lethal condition inherited as an autosomal recessive trait (Smith and Smyth, 1970).
**Causes of Clubbed Down**

Clubbed down was initially described in 1938 as a “French knot”. This “French knot” phenotype consisted of a buckling of feather plumules within the feather sheath resulting in a swelling in the mid-shaft portion of the feather. A riboflavin deficiency within the egg was the primary explanation for this “French knot” defect (Lepkovsky et al., 1938). Since that seminal report, other causes for clubbed down have been reported: nutrition and genetics (Lepkovsky et al., 1938; Hawes and Buss 1963).

Nutrition is believed to be one plausible explanation for the appearance of clubbed down. Riboflavin deficiency in the maternal diet results in riboflavin deficient eggs. Incubated riboflavin deficient eggs affect embryonic growth, hatchability and sometimes result in the appearance of clubbed down in the embryo (Lepkovsky et al, 1938; McClymont and Hart, 1947; Peterson et al. 1947; Brown, 1957, Korver, 2003). Deficient dietary levels of vitamin B_{12} in hen diets may also result in clubbed down of young embryos. Once deficient B_{12} levels were increased to an adequate level, clubbed down expression ceased (Ambrose, 2002). A low dietary zinc concentration in the diets of broiler chickens has also been associated with the appearance of clubbed down (Schuster and Hindmarsh, 1980).
Heredity is also a contributor to the expression of clubbed down as studies associating clubbed down in turkey embryos and the black plumage allele have shown (Hawes and Buss, 1963). This association led to the conclusion that the clubbed down condition in black turkey embryos resulted from a pleiotropic effect of the black color allele (Hawes and Buss, 1963). Bernier and Cooney, (1954) also reported that a higher incidence of clubbed down was also observed in chickens with a black plumage phenotype when compared with non-black chicks. It has also been found that a gene (Rd) in chickens prevented females from depositing sufficient riboflavin into their eggs. Embryos who inherit this riboflavin impairment gene had a greater incidence of clubbed down (Hawes and Buss, 1965). There are many variations in the expressivity of the clubbed down condition. In some situations, only one feather plumule is affected which is known as sparse clubbed down. In others, multiple feathers or even all the feathers contain the characteristic swelling resulting in severe clubbed down (Hawes and Buss, 1963).

The environment in which fertile eggs are incubated may affect the incidence of clubbed down. Because incubation temperature affects the rate of embryo development, it is possible that deviations from optimal incubation temperature may result in other embryonic disorders (Deeming, 2002). A high incubation temperature can accelerate embryo development.
Conditional Mutations

In addition to the commonly recognized categories of genes: structural, regulatory, temporal, etc., (Paigen, 1971) there is another less known category, the conditional gene. Klug et al. (2006) defines a conditional mutation or temperature sensitive mutation as a gene that is expressed normally at one (eg. normal) temperature but has an abnormal expression at a second temperature (eg. abnormal). If the gene’s expression is lethal at the abnormal temperature, that gene can be regarded as a conditional-lethal (Miglani, 2002).
CHAPTER 3
The Influence of an Elevated Incubation Temperature on the Expression of Clubbed Down in Coturnix Embryos

Introduction

Three down abnormalities localized to specific regions of the plumule in various avian species have been described and studied in developing embryos. In the proximal region of the turkey down plumule shaft, Savage et al., (1986) described the autosomal recessive lethal condition known as swollen down plumules. This condition was characterized by a swelling of the dermal pulp cavity of down feathers expressed between day 20 of incubation and time of pipping. At the distal end of the feather shaft also in turkeys, Smith and Smyth (1970) described this semi-lethal condition as knobby. The condition was characterized by an enlargement at proximal ends of the down. The knobby down was predominantly seen in the ventral feather tracts and this condition was the expression of an autosomal recessive gene when homozygous. In the mid-shaft region of the chicken’s feather shaft, ‘clubbed down’ was initially described by Lepkovsky et al., (1938) and attributed to a riboflavin deficiency. Since the initial clubbed down report, other nutritional deficiencies: zinc (Schuster and Hindmarsh, 1980) and B\textsubscript{12} (Ambrose, 2002) have been purportedly associated with clubbed down expression.
Genetic factors have also been attributed to the expression of clubbed down in turkeys and chickens (Hawes and Buss, 1963 and 1965; Bernier and Cooney, 1954). Recently, Colvin (2005) observed but did not investigate in a 10 generation genetic selection study for enhanced hatchability at an elevated incubation temperature, a significant incidence of clubbed down in Coturnix embryos. The following report describes studies performed to determine if incubation temperature and genetics were the basis for the expression of clubbed down in Coturnix embryos.
Materials and Methods

Matings and Incubation

The management of the breeders involved in this study have been described by Colvin (2005). The birds were all wing banded at hatching for pedigree identification. After the birds attained sexual maturity, single paired matings were placed in numbered cages and provided feed and water ad libitum. To study the clubbed down condition, eggs were collected and marked from each individual cage daily and the eggs were incubated weekly in one of two dedicated Jamesway 252 incubators (100 F and 102 F both with 56% relative humidity) and light candled on the seventh day of incubation. Eggs not exhibiting development were removed and macroscopic examination of the egg’s contents performed.

In attempting to determine if the clubbed down condition was heritable, matings were restricted to one type since the condition was lethal during embryonic development. The mating type consisted of carrier males mated to carrier females. Goodness of fit for categorical data obtained from the pedigreed matings were tested by individual, heterogeneity and pooled chi square analyses (Snedecore and Cochran, 1967).
In efforts to ascertain the influence of incubation temperature on clubbed down expression, eggs from Colvin’s (2005) control and selected lines were used in selected experiments.

**Comparison of eggs incubated at 100 and 102 F.**

To determine if incubation temperature, 100 F versus 102 F influenced the expression of clubbed down, eggs from five different pair matings of quail derived from the selected line were collected daily for 15 to 20 consecutive weeks and incubated weekly. The eggs from each setting were placed in either the 100 F or 102 F incubator and examined at 14 days for clubbed down. The assignment of the eggs to the differing incubator temperatures was at random, such that equal numbers of fertile eggs were incubated at each of the two temperatures.

**Incubation of eggs at 100, 102, and 103.5 F**

Additional eggs from Colvin’s control and selected lines were collected for 12 consecutive days and then immediately prior to placement in the incubators, the eggs were randomly assigned to one of three incubation groups, 100 F, 102 F, and 103.5 F. Equal numbers of eggs were present in the three groups and each set at a specific temperature and located within the same room. The 100 F and 102 F incubators were the same for all studies. Group 1 eggs were incubated at 100 F, group 2 eggs incubated at 102 F and group 3 eggs incubated at 100 F for seven
days and then transferred to an incubator set at 103.5 until day 14. On day 14, all eggs from the three groups were removed and their contents macroscopically examined for clubbed down.

**Scanning Electron Micrograph Preparation**

The preparation protocol of down plumule tissues for scanning electron microscope examination was described by Savage et al. (1986) with the following modifications. Plumule samples consisting of the plumules and attached epidermis were suspended in three rinses of deionized distilled water and moved through an increasing concentration series of aqueous ethanol; from absolute ethanol, the tissues were critical point dried according to Cohen et al. (1968) in a Balzers CP dryer; the prepared tissues were mounted on aluminum planchets using an adhesive and subsequently coated with approximately 200 Å of 60/40 gold-palladium alloy in a Edwards Sputter Coater; observations of the prepared tissues were made with an Amray 3300FE scanning electron microscope and images were digitally recorded.

**Histological Preparation of Samples**

Samples of down plumules (1 cm², consisting of the plumules and attached epidermis) were fixed in 10% neutral buffered formalin and processed in a Lynx Microscopy Tissue Processor. The processing schedule consisted of a tissue
suspension in 0.1M cacodylate buffer 2x 30 min RT; 1% osmium tetroxide in 0.1M cacodylate buffer 1 hr RT; 0.1M cacodylate buffer 2x 30 min at RT; transferring to an acetone series (10%, 30%, 50%, 70%, 80%, 95%, 100%, 100%, 100%) all 10 min at RT, 3:1 acetone: resin 30 min at RT, 1:1 acetone: resin 30 min at RT, 1:3 acetone: resin 30 min at RT, 100% resin 1 hr 30 C, 100% resin 1 hr at 30 C, 100% resin 20 hr at RT. The fresh resin was placed in flat embedding silicone molds, feather plumules were orientated longitudinally and the mixture polymerized for 24 h at 60 C. The resin was a modified Mollenhour (1964) formula of Epon-Araldite. (10 ml Embed 812, 10 ml Araldite 502, 24 ml DDSA, 0.9ml DMP-30 (Electron Microscopy Sciences, Fort Washington, PA.)). Plumule-embedded resin blocks were sectioned on a MT-5000 ultramicrotome using a glass knife. One micron sections were mounted on glass slides, dried, and stained with 1% toluidine blue.
Results

Characterization of Clubbed Down

The observed clubbed down is a bulbous or nodular swelling localized to the lower 1/3 of the plumule, and proximal to the epidermis (Figure 3.1). The typical affected down plumule exhibits a single projection or nodule though some plumules were observed to contain more than one nodule in close proximity to the primary lesion. The second nodule was smaller than the primary. The clubbed down nodules were more prevalent on the ventral surfaces of the embryo’s body than the corresponding dorsal surfaces (Figure 3.2). Clubbed down nodules were more prevalent in the pterylae of the neck and sternum than other feather tracks (Figure 3.3). Occasionally, clubbed down nodules were observed to be present in the head region but this was an uncommon observation. The number of clubbed down nodules per unit of body surface was variable among embryos, varying from 1 to 20+ in a 5mm² area. In some embryos, the nodules appeared red in color due to the presence of blood cells on the surface of the nodule. The clubbed down studied in this research has been identified in Coturnix embryos as early as 8 days of incubation. Embryos afflicted with the clubbed down condition can be smaller in body size than normal embryos of the same age (Figure 3.4). Some embryos exhibiting clubbed down may also have down feathers that appear coarser looking.
than a normal embryo’s down. Clubbed down embryos in this research have also been observed to exhibit other morphologic defects; e.g. amaxilla and ectopic viscera (Figure 3.5). In this study, some embryos with clubbed down have also been observed to be alive at 14 days of incubation and in normal embryo position (Figure 3.6).
Figure 3.1 Coturnix embryo exhibiting clubbed down at 12 days of incubation.
Figure 3.2 Appearance of clubbed down in a 14 day embryo. Top image shows ventral surface. Bottom image shows dorsal surface. Arrow shows the clubbed down.
Figure 3.3 Ventral surface of a 14 day old quail embryo exhibiting severe clubbed down. Arrow highlights the clubbed down in the majority of the feather tracts.
Figure 3.4 Variation in chick size among four full-sib Coturnix quail embryos incubated at 102 F and exhibiting clubbed down at 14 days of incubation.
Figure 3.5 Mid-dead quail embryo examined at 14 days of incubation exhibiting clubbed down (arrow), missing lower beak, and ectopic viscera.
Figure 3.6 Appearance of clubbed down embryos *in ovo* illustrating the variations observed – *(L-R;)* Left, Late dead (~ 16 days) with clubbed down; *center,* appearance of a Mid-dead (14 days) *in ovo;* right, embryo removed from egg.
Scanning Electron Micrographs (SEM) of Clubbed Down

The SEM micrograph (Figure 3.7) shows a normal plumule with its long, slender, flowing appearance and free from any projections and also a clubbed down plumule with the nodular/bulbous swelling in the distal third of the feather plumule shaft. A closer view at the nodular region (Figure 3.8) reveals that this region is composed of tiny bands of fibrous-like materials. An example of a plumule containing two nodules is illustrated in Figure 3.9. Figure 3.10 provides a clearer view of the interior of a nodule revealing that the content is devoid of cellular matter.
Figure 3.7 Scanning electron micrograph (55X) of clubbed down plumule from 13 day-old embryo. The lesion (arrow) is located mid-shaft and characterized by the absence of a sheath-like covering. The epidermis (EP) is to the left side of the down plumule. In this image is a normal down plumule (diamond).
Figure 3.8 Enlarged magnification (100X) of Figure 3.6 illustrating the fibrillar structure that appears to be present under the plumule sheath.
Figure 3.9 Clubbed down plumule with two bulbous-like structures (arrows) on the same structure, 100X.
Figure 3.10 Scanning electron micrograph of clubbed down plumule showing the absence of cellular matter within the center of the bulbous structure, 600X.
**Histological Description**

The histological examination of clubbed down plumules did not reveal any conspicuous lesions. “The portions of skin have evenly distributed feather follicles that are distinctly nodular. All components of the follicle were present. The follicular nodules were large, possibly hyperplastic, while producing normal feathers” (Heidel, 2006).

**Effects of Elevated Incubation Temperature**

The results of fertile eggs from five paired quail matings incubated to ascertain if incubation temperature influenced the expression of clubbed down are summarized in Table 3.1. Consistently throughout the experiment, no clubbed down was observed in embryos incubated at 100 F. Only in eggs incubated at 102 F was clubbed down expressed. There were no differences in fertility or early dead embryo mortality between eggs incubated at the differing temperatures. The results in Table 3.1 show that the 102 F incubation temperature was responsible for clubbed down expression. In further support of this determination, the results of a second study incubating different quail eggs at 100, 102, and 103.5 F are summarized in Table 3.2. In this study, the incidence of fertile eggs was lower than the preceding 5 trials (Table 1) because the eggs were stored for more than 7 days and hatchability declines with increased pre-incubation egg storage (Meijerhof, 1992). Only those eggs incubated at 102 F or 100/103.5 F
temperatures expressed clubbed down. Eggs incubated at 100 F for the first 7 days then 103.5 F from days 8-14, exhibited a two-fold increase in the incidence of clubbed down above the 102 F treatment. These results confirm that an elevated incubation temperature after the seventh day of incubation alters down formation. Savage (2004, unpublished observations) had made a similar observation regarding the incubation of avian eggs at 103.5 F. An explanation for the localized lesion however remains to be formulated.
**Table 3.1.** The effects of repeated incubation of fertile quail eggs from paired matings at either 100 F or 102 F on the incidence of clubbed down.

<table>
<thead>
<tr>
<th>Incubation Temperature</th>
<th>No. Eggs Set</th>
<th>No. Fertile</th>
<th>No Early Dead Embryos</th>
<th>No. of CD</th>
<th>% CD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trial 1</td>
<td>100</td>
<td>26</td>
<td>25</td>
<td>4</td>
<td>0 / 21</td>
</tr>
<tr>
<td></td>
<td>102</td>
<td>69</td>
<td>64</td>
<td>18</td>
<td>19 / 46</td>
</tr>
<tr>
<td>Trial 2</td>
<td>100</td>
<td>96</td>
<td>89</td>
<td>7</td>
<td>0 / 82</td>
</tr>
<tr>
<td></td>
<td>102</td>
<td>84</td>
<td>83</td>
<td>7</td>
<td>5 / 66</td>
</tr>
<tr>
<td>Trial 3</td>
<td>100</td>
<td>24</td>
<td>22</td>
<td>0</td>
<td>0 / 22</td>
</tr>
<tr>
<td></td>
<td>102</td>
<td>80</td>
<td>80</td>
<td>10</td>
<td>15 / 70</td>
</tr>
<tr>
<td>Trial 4</td>
<td>100</td>
<td>27</td>
<td>27</td>
<td>1</td>
<td>0 / 26</td>
</tr>
<tr>
<td></td>
<td>102</td>
<td>41</td>
<td>41</td>
<td>5</td>
<td>10 / 36</td>
</tr>
<tr>
<td>Trial 5</td>
<td>100</td>
<td>70</td>
<td>62</td>
<td>7</td>
<td>0 / 55</td>
</tr>
<tr>
<td></td>
<td>102</td>
<td>70</td>
<td>63</td>
<td>10</td>
<td>3 / 53</td>
</tr>
</tbody>
</table>

Trials: 1= August ’03; 2= December ’03, 3 = March ’04, 5 = June ’04, and 5 = July ’05

**Table 3.2.** The effects of incubating a population of fertile quail eggs randomly assigned to one of three incubation temperature regimes on the incidence of clubbed down.

<table>
<thead>
<tr>
<th>Incubation Temperature</th>
<th>No. Eggs Set</th>
<th>No. Fertile</th>
<th>No Early Dead Embryos</th>
<th>No. of CD</th>
<th>% CD</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>146</td>
<td>90</td>
<td>11</td>
<td>0</td>
<td>0 / 79</td>
</tr>
<tr>
<td>102</td>
<td>152</td>
<td>104</td>
<td>21</td>
<td>6</td>
<td>6 / 83</td>
</tr>
<tr>
<td>100-103.5</td>
<td>151</td>
<td>104</td>
<td>13</td>
<td>14</td>
<td>14 / 91</td>
</tr>
</tbody>
</table>

Temperature:

1 = 100 F day 1-14
2 = 102 F day 1-14
3 = 100 F day 1-7 and 103.5 F day 8-14
Environmental Interaction

Embryos incubated at 100 F had a normal appearance and normal numbers of chicks hatched alive. In embryos incubated at 102 F clubbed down occurred. For those embryos incubated at 103.5 F the incidence of clubbed down drastically increased. Based on the effect of increasing incubation temperature, clubbed down may be caused by a genetic x increased incubation temperature interaction.

Experimental Matings

Since the situation that clubbed down expression was influenced by incubation temperature (102 F) a genotype-environment interaction was present, attempts to identify and establish matings of individuals that would result in clubbed down individuals was difficult. Table 3.3 contains the results of three separate and unrelated matings of individuals that produced clubbed down offspring when eggs were incubated at 102 F. In all three separate matings, the ratio of clubbed down to normal embryos were consistent with 3:1 ratios, normal:clubbed down embryos when tested using chi-square (P>.05). In further support of the findings, when the data from the three matings were analyzed for heterogeneity and pooled chi squares, neither calculated value was significant (P>.05). Eggs from these same three matings were also incubated at the normal 100 F temperature and no
clubbed down embryos were observed. Efforts to establish matings of the proven carriers to possible non-carriers was not attempted. The advancing age of the breeders and the aggressive and usually lethal response that is elicited when paired matings are altered did not allow for that mating.

**Table 3.3.** Segregation data for three matings producing clubbed down embryos.

<table>
<thead>
<tr>
<th>Mating⁠¹</th>
<th>Normal</th>
<th>CD</th>
<th>Normal</th>
<th>CD</th>
<th>Calculated X²</th>
</tr>
</thead>
<tbody>
<tr>
<td>C29-G10</td>
<td>24</td>
<td>3</td>
<td>20.25</td>
<td>6.75</td>
<td>2.77</td>
</tr>
<tr>
<td>C40-G12</td>
<td>26</td>
<td>10</td>
<td>27</td>
<td>9</td>
<td>0.14</td>
</tr>
<tr>
<td>C7-G11</td>
<td>55</td>
<td>15</td>
<td>52.5</td>
<td>17.5</td>
<td>0.48</td>
</tr>
</tbody>
</table>

Discussion

Clubbed down appears to have a number of causes. Genetics, nutrition and the environment have all been mentioned as possibilities. Is it feasible that a combination of these may cause clubbed down? In this experiment, it appears that clubbed down is the result of an environment x genetic interaction. High incubation temperature caused a gene involved in the formation of feathers to function in an aberrant manner. This mutation results in the abnormal growth of embryonic down feathers resulting in the clubbed down condition. Nutrition does not appear to be linked to the appearance of the condition in the birds used in this experiment because all of the hens were fed the same diet. If there were a riboflavin, B₁₂ or zinc deficiency in the flock, all eggs would be affected. Nutrition has been mentioned as a cause of clubbed down in a number of other studies (Lepkovsky et al, 1938; McClymont and Hart, 1947; Peterson et al. 1947; Brown, 1957, Schuster and Hindmarsh, 1980; Korver, 2003). Other studies refer to a gene (E) for black plumage color in chickens to be associated with predisposing embryos under the appropriate conditions to clubbed down (Hawes and Buss, 1963). In Coturnix quail, variations in plumage phenotypes are few since most quail exhibit a wild-type phenotype with considerable black melanin probably inferring the presence of the E-type gene, similar to chickens. It may be
possible that clubbed down is the result of a number of mutations that produce the same phenotype. In our case, a genetic x environment interaction appears to be the cause of clubbed down, but in many other studies it may have been a nutritional deficiency or gene that produced the mutation resulting in the expression of the clubbed down phenotype.

In this study, it appears that a lethal temperature sensitive mutation has been identified and may be an explanation for the clubbed down observed in the Coturnix quail. In the literature relevant to clubbed down observations in poultry, there have not been studies of incubation temperature. It appears from the studies described here that temperature may be influencing the action of either an enzyme/s associated with feather plumule development and or utilization of perhaps vitamins that serve as metabolic cofactors in essential metabolic pathways.

Based on the findings in this study, the gene for clubbed down appears to be temperature sensitive. Under normal incubation temperatures this temperature sensitive gene functions normally, down feathers in the embryo grow in a typical fashion. Under higher incubation temperatures this temperature sensitive gene is affected and results in improper feather development, clubbed down.
The three feather abnormalities: knobby, swollen down and clubbed down listed in this paper have similar outward appearances. All are bulbous-like swellings within the feather shaft. It is when we examine the internal structure of these swellings that differences can be observed. In both the knobby and swollen down conditions the swellings are filled with loosely disorganized tissue (Smith and Smyth, 1970; Savage et al., 1986). Based on the SEM and histological findings, clubbed down appears to be devoid of any internal cellular matter. Because these swellings look so similar it seems possible that they are related, but because the internal structures are different there may be no relationship at all. The relationships and differences in these conditions may be something to look at in the future.

Heat-shock proteins are produced by cells in response to extreme heat. Heat shock causes activation of a small number of inactive genes and inactivation of some previously active genes and selective translation of heat-shock mRNA (Klug et al., 2006). Could clubbed down be the expression to a heat-shock protein? This is something that we do not know, but based on the definition of heat-shock proteins it appears that this could be a possible explanation for the clubbed down condition. A high incubation temperature could be shocking certain genes to turn on or off resulting in a mutation in the feather shaft.
These findings bring about other questions involving the exact cause of the clubbed down condition. What is happening to this gene when the mutation occurs? What enzymes are involved? How are knobby, swollen down and clubbed down related? Further studies concerning clubbed down at the molecular level could help in explaining how high incubation temperature affects the gene involved in the development of this condition.
LITERATURE CITED


