CZECH UNIVERSITY OF LIFE SCIENCES PRAGUE

Faculty of Agrobiology, Food and Natural Resources

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Interactions of housing system, genotype and eggshell quality and their relationship to egg safety

Ph.D. Thesis

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Prague 2018

Declaration

I declare that I have elaborated my thesis titled **"Interactions of housing system, genotype and eggshell quality and their relationship to egg safety"** on my own with a help of literature listed in References.

The study was supported by the Ministry of Agriculture of the Czech Republic (Project No. QJ1310002).

In Prague date

Acknowledgments

I would like to express my deepest thanks to Prof. Ing. Eva Tůmová, CSc. For her valuable advices and professional guidance to complete this work.

I would like also to thank Ing. Darina Chodová, Ph.D., Ing. Jana Vlčková, Ph.D. and all my colleagues at the department of animal husbandry for four years of help and assistance.

My lovely family Doc. Hammad Ketta, Ph.D. and good friends Prof. Tarek Ebeid, Ph.D. and Dr. Said Ali for their patience and support.

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1. Introduction

Eggshell quality is one of the major problems facing poultry industry causing a huge impact on the profitability of egg production. The shell of the egg is a natural envelope which protects the developing embryo against physical damage.

The eggshell layers are deposited sequentially as the egg passes through the different regions of the oviduct. Laying hen eggshell represents about 10% of total egg weight. It consist of two main parts; shell membranes and true shell. The true shell includes mammillary layer, palisade layer, vertical layer and cuticle. The eggshell structure constituted 96% of calcium carbonate and an organic matrix composed of proteins, glycoprotein and proteoglycans (3.5%) with assortment of microelements. This structure is the consequence of controlled interactions between both mineral and organic matrix constituents to give the final complex bio-ceramic.

Studies expressed the quality of the eggshell through its weight, percentage, thickness and strength. These properties are affected by wide range of factors which combine to improve the final product. The internal factors include time of oviposition, age and genotype; and the external factors including housing system, nutrition, environmental conditions, lightening regime and stress. All these factors are known to influence the eggshell quality characteristics individually, as well as interactions between some of these factors could be more effective.

Housing systems for laying hens take place in conventional cages, because it is the most economic housing system regarding to higher number of birds housed in limited space. However, conventional cages has alarmed public concern for intensively housed birds, which began to increase, with new animal protection laws came into force producers to adapt to the welfare concerns of consumers, which is oriented towards healthy foods controlled not only under a safety point of view, but also under a welfare assessment of the animal's living conditions. Therefore, there was a trend to develop and use barn housing systems for laying hens to fulfill some of their natural behavior reflecting eggs with better quality. However, according to the differences between housing systems the eggshell properties also differed in its weight, percentage, thickness and strength.

Cuticle deposition is an important factor against microbial penetration into the eggs, as cuticle layer contain substances with anti-microbial activity such as lysozyme. Therefore, maximizing the cuticle coverage onto the egg is very important issue for egg safety and consequently profitability.

2. Literature review

2.1. Eggshell structure and formation

Research interest in eggshell quality is ongoing event, because the eggshell is very important to resist physical and pathogenic challenges from the external environment; also it is an important concern for consumers, as strong resistance to breaking and lack of shell defects are essential for protection against the penetration of pathogenic bacteria into eggs. Eggs with shell defects ensure significant economic losses at different stages of the egg production process. Therefore, understanding the eggshell structure and factors affecting it will be of benefits to maximize the production and profitability.

The interest about the eggshell structure had been started earlier in the nineteen century by Von Nathusius who well-defined the structural polycrystalline organization. Recently, several studies have been conducted on the structure of the avian eggshell (Nys et al., 2004; Nys and Gautron, 2007; Rodriguez-Navarro et al., 2007; Hincke et al., 2010, 2012; Gautron et al., 2014).

Five hours after ovulation, the forming egg enters the red isthmus and uterus where the eggshell calcification occurs during a period of 18-19 hours. During mineralization, the un-complete egg bathes in a cellular milieu (the uterine fluid) that contains ionized calcium and bicarbonate which is necessary for the eggshell formation. The process done by controlled precipitation of calcium carbonate on the outer eggshell membrane fibers, and occurs in the extracellular space between the dilated shell membranes that envelope the hydrated albumen and the mucosa of uterine wall (Hincke et al., 2012; Gautron et al., 2014). The uterine fluid changes in composition during different stages of the eggshell formation and influences calcite crystal growth in different zones of the calcified shell (Nys et al., 2004).

Mineralized eggshell is about (96%) calcium carbonate; the remaining components include the organic matrix (2%) as well as magnesium, phosphorus and a variety of trace elements (Nys et al., 2004). From the inside outwards, the eggshell comprises of shell membranes and true shell that includes mammillary layer, palisade layer, vertical layer and cuticle (Hincke et al., 2008; Gautron et al., 2014). The eggshell membranes are a fibrous structure situated between the eggshell and egg albumen. It is essential for the formation of the eggshell and it also provides the shell foundation except at the blunt pole of the egg where they separate to form the air-space. The eggshell membranes are secreted and assembled during approximately one hour, resulting meshwork of interlaced fibers composed of roughly 10% collagen and 70-75% of other proteins and glycoproteins containing lysine-derived cross-links which

organized into morphologically distinct inner and outer sheets that enclose egg albumen (Hincke et al., 2012). The total thickness of these two membranes has been found at approximately 100 μm. Each of these membranes is composed of protein fibers that are arranged so as to form a semi-permeable membrane. The inner membrane remains un-calcified, while the fibers of the outer shell membrane become mineralized at discrete sites and become incorporated into the base of the eggshell (Nys et al., 2004). A thin inner film is the most interior barrier of the eggshell, and it is overlaid by the proteinaceous inner and outer shell membranes, shell membrane with the thin inner film impede oxygen diffusion to the same extent as does the shell proper; whereas, the shell proper is totally responsible as the barrier for both carbon dioxide and water vapor diffusion.

Specific nucleation sites on the outer surface of the outer shell membrane attract calcium salts and so initiate the formation of the mammillary layer in that region of the oviduct termed the tubular shell gland (Solomon, 2010). The mammillary cones are small masses of organic matter that represent the seeding sites on which crystallization of the shell begins, these cones are penetrated by fibers of the outer eggshell membranes.

The mammillary cones are exclusively the main source for calcium mobilization during embryonic development (Karlsson and Lilja, 2008; Chien et al., 2009). Therefore, mammillary core formation and distribution are related to the mechanical strength and respiratory quality of the eggshell (Robinson and King, 1970; Koga et al., 1982). Pores formation begins at the level of the mammillary layer with the grouping of 4-5 mammillary bodies. As they grow laterally and vertically, their orientation is such that a central space is left which in functional exchange sites persists through the entire depth of the shell (Solomon, 2010).

From and over the mammillary layer the palisade layer develops as the main layer of the shell, this layer comprises about 200 μm as the thickest aspect of the shell, where the calcite crystals grow with a long aspect perpendicular to the surface. The mammillary layer is the site of a range of structural defects which can be reduced through organically bound selenium (Solomon, 2009). Recently, increased chemical reactivity has been found in nano-selenium (Suchý et al., 2014). Palisade columns grow from one mammillary knob and as the calcification mechanism proceeds adjacent columns fuse. This layer ends at the vertical layer which has a crystalline structure of higher density than that of the palisade layer (Hincke et al., 2012).

The eggshell cuticle is an uneven organic layer covering the outer surface of the eggshell. It is composed of inner calcified and outer non-calcified water insoluble layers which are

deposited directly onto the vertical crystal layer of the eggshell (Rose and Hincke, 2009; Kusuda et al., 2011). Unfortunately not all avian eggs have a cuticle layer, and the distribution of cuticle is often patchy (Bain et al., 2009; Samiullah and Roberts, 2014).

The pores system of the avian eggshell is located at specific locations between the eggshell cones (columns, prisms) to provide gas and humidity exchange. In chicken eggs, typical pores have a funnel-shaped orifice opening at the outer shell surface at the level of the cuticle, and a single channel passing through the vertical crystal layer and the palisades region to open at the inner surface of the eggshell between neighboring mammillae (Chien et al., 2008).

The shell structure might have a significant effect on eggshell characteristics mainly thickness and strength. Bain (1991) suggested that the organization of the palisade columns in addition to crystals size and its orientation is a major determinant of shell thickness and strength. Therefore, it is likely that changes in the thickness of the palisade layer independent of structural re-organization of the palisade columns could affect shell strength. Rodriguez-Navarro et al. (2002) revealed a correlations between eggshell strength and crystallo-graphic texture. Authors concluded that, about 40% of the variance in shell strength could be explained by differences in the degree of orientation of crystals.

Eggshell deposition occurs in three stages coincide with sequential secretion of organic matrix constituents in the cellular uterine fluid with the rate of calcium carbonate deposition (0.32 g/hour) as the fastest known bio-mineralization event (Nys et al., 1991). The entire process lasts around 17 h in the highly selected breeds as layers, and considered as the longest phase of egg formation (Nys et al., 2004). The first stage is about 5 hours in duration and corresponds to the initiation of mineralization. The first crystals of calcite are nucleated at the sites of the organic aggregates present on the surface of outer shell membranes (Hincke et al., 2012). Distribution of these nucleation sites is under genetic control and varies among species. The second stage corresponds to the growth phase and lasts about 12 hours (Gautron et al., 2014). It is an active calcification phase forming the compact calcified palisade layer (2/3 of the total thickness of the shell), which extends beyond the bases of the cones and ends in the vertical crystal layer. The last stage corresponds to termination of calcification and lasts about 1.5 hour (Nys et al., 2004). It is characterized by the arrest of mineralization and deposition of the organic cuticle which covers the entire surface of the egg (Hincke et al., 2010, 2012; Gautron et al., 2014).

Minerals of the eggshell are associated with an organic matrix of soluble and insoluble proteins, glycoproteins, and pro-teoglycans, representing about 2% by weight of the calcified eggshell which are progressively incorporated from the uterine fluid during calcification

(Hincke et al., 2010). The importance of eggshell matrix proteins is related to influencing the foundation of the eggshell and participates in antimicrobial defenses (Hincke et al., 2012; Gautron et al., 2014). Gautron et al. (2014) reported that, the eggshell matrix components have been divided into three groups according to their origin. The first group is composed of egg proteins originally characterized in the egg white (ovalbumin, lysozyme and ovo-transferrin). They are mainly localized in the basal parts of the shell (eggshell membranes, mammillary cone layer), but also cuticle (Gautron et al., 2001; Hincke et al., 2010; Gautron et al., 2014), and are mainly associated with the initial phase of shell calcification in the uterine fluid. The second group is made of proteins that are widely found in various organs and biological fluids. This group includes osteopontin (a phosphorylated glycoprotein of bone, kidney and present in various body secretions and also present in the core of the non-mineralized shell membrane fibers, and in the outermost part of the palisade layer of the chicken eggshell) and clusterin which a widely distributed secretory glycoprotein also present in the egg white (Gautron et al., 2014; Brionne et al., 2014). The third group named eggshell-specific proteins because it were identified during investigation of abundant constituents of the eggshell and uterine fluid. These components were termed as ovocleidins and ovocalyxins (Hincke et al., 2012; Gautron et al., 2014). Two possible roles for the Ovocleidins and Ovocalyxins have been proposed in avian reproduction: regulation of eggshell mineralization and anti-microbial defense (Gautron et al., 2001; Hincke et al., 2012).

Gautron et al. (2014) reported a number of experimental observations support the critical role of the eggshell matrix proteins in determining the fabric of the eggshell and its resulting mechanical properties. The first is related to the chicken eggshell matrix with its content of relatively specific proteins (ovocleidins and ovocalyxins), of which mRNA is strongly expressed and proteins synthesized at high levels in tissues where eggshell calcification takes place, namely, the red isthmus and uterus (Gautron and Nys, 2007a). The second experimental evidence is the change in the protein composition of the uterine fluid during the progressive fabrication of the eggshell. The uterine fluid of each phase of shell mineralization has a unique protein electrophoretic profile, suggesting that they possess specific roles during the calcification process (Gautron et al., 1997). The nature of the interactions between matrix components and the mineral phase of the shell has been carefully investigated using *in vitro, in situ* and genomics approaches (Gautron and Nys, 2007b, Hincke et al., 2010; 2012; Gautron et al., 2014).

2.2. Eggshell quality properties and its measurements

The quality of the eggshell has been monitored in the long term for purposes of selective breeding. Numerous parameters have been proposed to evaluate eggshell quality in order to reduce the losses of damaged eggshells, that parameters include eggshell weight, percentage, thickness, strength and density. Eggshell quality can be measured by various methods; some of these methods require destruction of the egg, in addition, some methods are direct whereas others are indirect (Roberts, 2004). Direct methods include measuring of shell breaking strength such as impact fracture force, puncture force or quasi-static compression. Indirect methods include specific gravity, non-destructive deformation. However, in commercial operations, eggs are either candled using light to detect cracks and other defects or they pass through an electronic crack detector for egg breakage detection.

For several decades, scientists spared no effort to find a new effective techniques or instruments to evaluate eggshell thickness and strength to reduce the economic losses of damaged eggshells. Sun et al. (2012) introduced a new parameter called uniformity of eggshell thickness to evaluate eggshell quality. Authors defined it as the reciprocal of coefficient of variation of eggshell thickness from multiple positions and obtained that, uniformity of eggshell thickness had a significant positive correlations with breaking strength which provided a new tool for evaluation of eggshell quality. Moreover, Yan et al. (2014) studied the relationship between the uniformity of eggshell thickness and eggshell quality of Lohmann Brown eggs and reported that, the uniformity of eggshell thickness is positively correlated with eggshell thickness (0.297), breaking strength (0.430), static stiffness (0.409), and fracture toughness (0.171) which might be used as an important indicator for other shell measurements in poultry breeding. Kibala et al. (2015) developed a new methodology using ultrasonic technology to record eggshell thickness at different egg latitudes. The authors observed genetic correlations between eggshell strength and its thickness to be around 0.8, making shell thickness a selection index candidate element. In their study, interrelationships between morphological, densitometric and mechanical properties in Japanese quails eggs Tatara et al. (2016) used dual-energy X-ray absorptiometry, quantitative computed tomography and three-point bending test and they found positive correlations between weight, height and width of eggs and egg mineral content and egg volume. Moreover, the mean volumetric eggshell mineral density was positively correlated with eggshell breaking strength and negatively correlated with eggshell thickness. The authors concluded that the elaborated experimental model used in the study may serve for further investigations on physiological,

pharmacological, environmental, nutritional and toxicological factors influencing egg quality not only in Japanese quails but in other bird species as well.

Cuticle estimation is an important issue regarding to its function to prevent micro-organisms penetration. The most popular method for cuticle estimation is an individual intact of an egg with a suitable stain such as MST cuticle blue stain (MST Technologies, Europe Ltd) for one minute and then rinsed two to three times in tap water. MST cuticle blue stain is a reliable indicator of the amount of cuticle present on an eggshell. Then the eggshell surface color can be measured using a Konica Minolta hand-held spectrophotometer (CM-2600d) (Messens et al., 2007; Leleu et al., 2011; Roberts et al., 2013; Samiullah et al., 2014).

2.3. Housing systems of laying hens

Over the last few decades, new laying hen housing systems have been introduced in an effort to harmonize poultry health and welfare with consumer, producer, industry and environmental demands. Following Council Regulation 1999/74/EC, from 2012 conventional cages are prohibited in the European Union (EU) and laying hen housing systems had to change from (mainly) conventional cages to furnished cages and non-cage systems (Rodenburg et al., 2005; Tauson, 2005).

2.3.1. Cages housing systems

In cages housing systems the laying hens are kept in mesh wire cages without any type of outside access. However, there are two types of cages that have been reported in literatures; conventional cages and enriched cages.

In conventional cages a small group of hens is kept in an enclosure of welded wire mesh with a sloping floor. The system enables the farmer to keep a large number of birds in a restricted building space, but yet to keep them in small groups. The space allowance varies from 400-750 cm^2/hen (Hester, 2005). The advantages of conventional cages are represented in better performance of layers, including higher egg production, better feed conversion ratios, and lower mortality (Leyendecker et al., 2001a; Voslářová et al., 2006). Conventional cages also limits the sanitary problems, reflecting better health indicators and lower number of dirty eggs (De Reu et al., 2009) and higher egg production (Englmaierová et al., 2014). On the other hand, there are serious welfare disadvantages because of the lack of freedom of movement, comfort, shelter, suitable flooring and freedom to perform most of natural pattern of behavior (Blokhuis et al., 2007).

The enriched cages is relatively new housing system for layers; it has been developed with the aim of giving laying hens the opportunity to perform certain natural behavior with relevant resources like nests, perches and litter area, while, maintaining the economic and hygienic advantages associated with cages (Tauson, 2005). The provisions of Regulation 1999/74/EC (CEC, 1999) stipulates a minimum space of allowance at least 750 cm^2 area/hen of which 600 cm^2 shall have 45 cm free height above the area. In addition the cage shall have a nest with no direct contact to any wire mesh floor, 15 cm of perches/hen, a feed trough of 12 cm/hen. Fiks-van Niekerk and Elson (2005) suggested a distinction between three categories of enriched cages: small (up to 15 hens), medium (15-30 hens) and large (more than 30). A number of studies reported the advantages of enriched cages represented in better shell quality compared to eggs from conventional cages (Karkulín, 2006). Enriched cages considerably improved the welfare of hens (Pohle and Cheng, 2009) and the hygiene of eggs (Roll et al., 2009) and did not differ in egg weight (Tactacan et al., 2009).

2.3.2. Barn housing systems

Improving rearing methods holds a great promise for the positive effect on performance and welfare of laying hens in non-cage systems. Among both scientists and practitioners, there is increasing agreement that the rearing environment should match the laying environment as closely as possible. Barn systems for laying hens have provided greater freedom of movement and facilities for natural behavior of birds including the use perches and nests (Tauson, 2005). On the other hand, several recent studies have demonstrated that hens housed in non-cage systems have a higher risk for increased injuries and mortality compared with cage systems (Sherwin et al., 2010; Lay et al., 2011; Rodenburg et al., 2012). However, there are three types of barn systems; aviary, litter and free-range.

Aviaries are multi-tier systems that consist of a littered ground floor and a metal structure with up to four tiers. A portal-type aviary provides a single level on top of 2 stacks (Tauson, 2005). In various countries, this new housing system has been assessed with regard to animal welfare health and economic aspects. According to EU Regulation 1999/74/EC, up to 18 hens/m^2 house floor area can be kept, with a stocking density of 9 hens/m^2 on the useable area (Heerkens et al., 2015). Aviaries vary in design, although all systems typically have feeders, drinkers, and perches located on one or more tiers. Access to the ground floor and multiple levels increases the surface accessible to the hens and allows them to perform natural behaviors such as running, wing flapping, flying, nesting, perching, and dust bathing when

compared with cage environments (Leyendecker et al., 2005). So that, one of the aviary's best features is the construction of tiers and perches above the littered area, which allows the hens to move and get out of the way both horizontally and vertically. Furthermore, the spatial separation of the areas for eating, drinking, resting, foraging and dust bathing is considered as advantageous (Fröhlich, 1995). As disadvantages of aviary systems, Aerni et al. (2005) found that, aviary hens consumed more feed and produced less egg mass per hen housed. The feed conversion ratio, as a result, was poorer in aviaries than in cages. These differences in feed consumption may be due to differences in feed intake or feed wastage. Moreover, Rodenburg et al. (2008) reported higher mortality and lower performance.

Litter housing system is one of the oldest systems for keeping hens, with simple floor area. Litter systems can vary considerably in design and layout depending on the type of building. The classic form consists of 80-90 cm high dropping pits covered with sand, straw, wood shavings or other materials gives the hen room for moving about and scratching. Feeders, drinkers and laying nests should be positioned on top of the dropping pit and the drinkers should be mounted at a distance of 30 to 50 cm directly in front of the entrance to the nest. A litter system has a lot of disadvantages represented in higher daily feed wastage. Egg productivity also is negatively affected by litter system, as increased number of lost eggs in the floor area; moreover the higher number of cracked eggs, in addition to the negative effect on eggshell cleanliness because of houses sanitation problems (Roll et al., 2009). Providing litter systems with perches is a way to relieve laying hens stress and to reduce certain injuries and cannibalism. Perches can play a role in manure management as well. Perches allow birds to stay off the floor, particularly during the night. Consequently, manure tends to accumulate under the roost area, and the rest of the bedding material in the house stays cleaner.

Free-range systems allows hens to spread out to preferred distances when foraging, typically greater than 5,000 cm^2/hen (Savory et al., 2006), and greatly expands behavioral options, especially if the range offers a variety of plant types. Access to an outdoor run gives the hens some natural light and fresh air with increased opportunities for foraging behavior, for which laying hens are strongly motivated (Dixon, 2008). Daylight may also be very important for behavioral development and general performance.

Two new housing systems could be added to free-range system for optimal hen performance and production; winter garden and Rondeel systems. A winter garden attached to the poultry house has proved highly beneficial. The hens cross the winter garden to get to the outdoor enclosure. Winter gardens in front of the laying house have a positive effect on both litter

quality and house climate: when the pop-holes are opened, cold air does not flow straight into the building and the indoor temperature is less affected than without a winter garden (Thiele and Pottgüter, 2008). The advantages of winter garden compared with normal free-range systems are that, the winter garden could be completely enclosed and used by the hens all year round including the cold seasons providing the ability to control the environmental conditions including temperature and humidity.

The Rondeel system provides the hens with a large, indoor pecking and scratching area on artificial grass with ample daylight and a smaller forest which can be closed in case of health or food safety risks (Rodenburg et al., 2008). The Rondeel is a circular building that can house 30,000 hens in 6 sections, located around the central management quarters. Egg collection is located in these management quarters; also a manure drying tunnel is situated below ground level there. The Rondeel advantages aims to combine issues like animal welfare, environmental care and consumer demand. Although the wooded fringe does not meet the requirements for free-range, it does provide the birds with range possibilities, without any risk of predators. Also it is easier to control and disinfect than large areas free-range. In cases of infectious diseases and the necessity to lock birds in the henhouse (Fiks-van Niekerk and Reuvekamp, 2011). The Rondeel disadvantages represented in more expensive to build than traditional aviary or free-range houses and therefore egg production costs are higher.

2.4. Factors affecting eggshell quality

Eggshell quality is influenced by wide range of factors which combine to influence the final product. The internal factors include for example time of oviposition, age and genotype; and the external factors including housing system, nutrition, microclimate and etc. All these factors are known to influence the eggshell quality characteristics, as well as, interactions between some of these factors could be more effective than individual factors.

2.4.1. Internal factors influencing eggshell quality

Time of oviposition plays a vital physiological role in determining eggshell characteristics, because the amount of deposited shell is a linear function of time spent in the shell gland after plumping. The distribution of oviposition times in laying hens is restricted to an 8 h period of the day with eggs being laid normally between 7:30 and 16:00 h under standard lighting conditions (Campo et al., 2007).

The oviposition time significantly affects the eggshell weight, which was higher of eggs laid before 07:45 h than eggs laid between 07:45 h and 11:45 h (Harms, 1991). Then, shell weight significantly increased until 12:45 h and remained greater through the rest of the day with exception of eggs laid between 14:45 h and 16:45 h. Tůmová et al. (2009) described a declining trend in shell weight with collection time especially in Isa Brown genotype with values of 6.38 g at 06:00 h and 6.23 g at 14:00 h. On the other hand, Tůmová and Ebeid (2005) and Tůmová et al. (2007) indicated that, eggshell weight was higher in the afternoon eggs at (14:00 h). Therefore, it might be assumed that eggshell weight tends to increase at the terminal egg of the clutch.

Oviposition time may also affect the eggshell thickness as an important indicator for eggshell quality. Yannakopoulos et al. (1994) assumed that, higher shell quality is due to thicker shell in the afternoon eggs. These results are in agreement with the finding of Tůmová and Ebeid (2005) and Tůmová et al. (2007) who indicated that, eggshell thickness of eggs laid in the morning is not as good as those laid in the afternoon. Contrary, Tůmová and Ledvinka (2009) revealed significantly higher eggshell thickness in the morning (06:00 h) and decreased with the collection time which might be affected by genotypes used in their experiment. Moreover, the eggshell quality can be affected by the content of minerals in the eggshell. Tůmová et al. (2014) reported a great effect of oviposition time on shell mineral content with the highest Ca content (352 g/kg) in eggs laid at 07:30 h and (342 g/kg) at 15:30 h. On the other hand, the P and Mg shell content increased with late oviposition time with the values of (1.20 and 3.56 g/kg; respectively) at 07:30 h and the values of (1.43 and 3.88 g/kg; respectively) at 15:30 h. The higher shell Ca content in early morning eggs is related to higher rates of Ca deposition in modularly bones during the dark period as it was assumed by Kebreab et al. (2009).

The eggshell characteristics might vary in different stages of laying hens age. Very young birds with immature shell glands produce shell-less eggs or eggs with a thin eggshell. The founding of Tůmová and Ledvinka (2009) indicated that, eggshell weight increased with hens age. The heaviest eggshells (6.67 g) were found at the age of 56-60 weeks in comparison with (5.05 g) at 20-24 weeks of age. Similar findings in layers and broiler breeders were documented by Tůmová et al. (2014). Increasing the eggshell weight with aged hens is related to the increasing size of the egg and shell surface area.

Bozkurt and Tekerli (2009) found out a decreasing of shell thickness with advancing age. In different study by Tůmová and Ledvinka (2009), thicker eggshell (0.372 mm) at the age of 56-60 weeks in comparison with 20-24 weeks of age (0.354 mm).

Eggshell strength as a function of other eggshell measurements is the most important measurement for egg producers; because lower strength causes higher percentage of broken eggs increasing the economic losses. Zita et al. (2009) observed that, eggshell strength was improved from the onset of lay till the end of the first phase and afterwards declined. However, Pavlik et al. (2009) indicated a decreasing of eggshell breaking strength with the age of birds; they reasoned it to higher plasma mineral content with aged hens. Similarly, Tůmová et al. (2014) detected a decreasing in eggshell strength in older hens (3.33 kg/cm^2) in comparison with the younger ones (3.60 kg/cm^2). In addition, the hens age also affect the egg specific gravity as an indicator for eggshell thickness and strength. Tůmová and Gous (2012) reported a decreasing in specific gravity with hen age. The differences among papers in eggshell quality and hens age can be related to genotype and the conditions of the experiments.

Marked differences in eggshell quality follow from the particular breed, line and family of the laying hens. Therefore, it is important to select an appropriate genotype respectively to improve eggshell quality through genetic selection. Among eggshell quality characteristics; eggshell weight, thickness and strength have a great differences between white and brown eggs. Hocking et al. (2003) reported that, in contrast to changes in egg weight during hens selection, eggshell weight did not changed. Similarly, Singh et al. (2009) observed that, eggshell weight did not differ between Lohmann White and Lohmann Brown. Both hybrids produced heavier eggshells then H&N White genotype. There are also differences in eggshell weight within brown hybrids. Tůmová et al. (2011) found the heaviest eggshells in Isa Brown (6.3 g) in comparison with Hisex Brown (6.1 g) or Moravia BSL (5.5 g). Similar results were reported by Ledvinka et al. (2012). All these results correspond with findings of Hocking et al. (2003) who described that genetic correlations within commercial hybrids for eggshell weight are 0.63.

Eggshell thickness is related to length of eggshell formation and is more affected by genotype in comparison with eggshell weight and probably more reliable indicator of the eggshell quality than eggshell weight. Differences between white and brown hybrids in eggshell thickness are described by Ledvinka et al. (2000) and Leyendecker et al. (2001a). Authors found thicker shells in brown hybrids. Within brown hybrids, similar results of the eggshell thickness were found as in eggshell weight. The thinnest shells were observed in Moravia BSL (0.324 mm) in comparison with Isa Brown (0.376 mm) or Hisex Brown (0.358); (Tůmová et al., 2011; Ledvinka et al., 2012). Tůmová et al. (2007) compared three Dominant

genotypes, Plymouth Rock strain, Blue strain and their cross. Plymouth Rock strain produced thicker eggshells in comparison with Blue strain and their cross had eggshell with average thickness of both strains. The results correlate with Hocking et al. (2003) that selection of commercial hybrids does not change the thickness of the shell.

The eggshell weight and thickness are physical variables which correlate with eggshell strength. Higher shell strength was revealed in white egg chicken in comparison with the brown ones (Ledvinka et al. 2000). Non-significant differences in shell strength were determined by Tůmová et al. (2007) in variable Dominant strains. However, in experiments with brown hybrids Isa Brown, Hisex Brown and Moravia BSL; significantly stronger shells were observed in Isa and Hisex Brown (Zita et al., 2009; Tůmová et al., 2011; Ledvinka et al., 2012). The contrast results of the eggshell strength might be related to low heritability of eggshell strength (0.24; Zhang et al., 2005). Eggshell quality measurements have low heritability and are more affected by environmental factor; however, correlations between individual characteristics and eggshell strength are more important. Frank et al. (1965) indicated that differences in the physical variables like eggshell weight and thickness can explain nearly 60% of the eggshell strength variation.

2.4.2. External factors influencing eggshell quality

Housing system is one of the main external factors that influence the eggshell quality. Several studies have been done in order to evaluate the effect of housing systems on eggshell quality parameters including conventional cages, enriched cages, litter and free-range systems. Lower number of cracked eggs has been produced in cages (Tůmová and Ebeid 2005; Holt et al., 2011; Kontecka et al., 2014). Different eggshell weights have been reported in literature according to housing systems; Pištěková et al. (2006) detected heavier eggshells in cages (8.11 g) than on deep litter (7.71 g). Moreover, heavier eggshells in un-enriched cages in comparison with floor system and enriched cages were obtained by Lichovníková and Zeman (2008). In contrast, Tůmová et al. (2011) detected heavier eggshells on litter than in conventional cages and enriched cages. These contradictory results are presumably related to different environmental conditions among housing systems; in addition to different hen genotype used in the experiments.

Eggshell thickness also varies according to housing systems. Comparing litter, free-range and cages housing systems, Pavlovski et al. (2001) detected thicker shells on litter eggs and thinner shells in free-range. Marked differences between cages and free-range systems in eggshell thickness were described by Leyendecker et al. (2001b) and Hidalgo et al. (2008).

They found lower eggshell thickness in eggs produced in cages while free-range eggs presented the highest values. Moreover, the differences between cages and litter were reported by Ledvinka et al. (2012). The authors found thinner eggshells in cages (0.355 mm) in comparison with litter (0.358 mm). These results are in correspondence with Mostert et al. (1995) who found greater eggshell thickness in eggs from non-cage systems.

Major economic losses for egg producers are consequences to lower eggshell strength which results in eggshell breakage. Mertens et al. (2006) examined the effects of multiple housing systems (conventional cages, enriched cages, aviary, and free-range) on eggshell quality and reported that, shell strength was the greatest in aviary eggs and the weakest in free-range eggs. Moreover, an experiment by Tůmová et al. (2011) conducted on the effect of housing system on eggshell strength resulted stronger eggshell produced in cages housing system (4744 g/cm^2) compared with litter (4651 g/cm^2). Similarly, Ledvinka et al. (2012) and Englmaierová et al. (2014) found stronger shells in cages than litter. However, non-significant differences in shell strength between eggs from the deep litter system and cages were reported by Pištěková et al. (2006). These results could be either affected by hen genotype or different experimental conditions. However, in spite of the shell thickness was lower in eggs produced in cages Tůmová et al. (2011) and Ledvinka et al. (2012) found higher eggshell strength. The authors reasoned it to the ultra-structural features of the shells in cages eggs which presumably support the eggshell strength. Moreover, Ketta and Tůmová (2018) reported that eggs with the thickest shells from enriched cages had significantly stronger shells than those from litter system. These results indicated that in the thin shell thickness, housing system plays an important role in the relationship to strength.

Nevertheless, it might be assumed that housing system affect eggshell microstructure resulting different eggshell thickness and strength. The assumption is also related to the effect of housing system on pores density. Significant effect of housing system on eggshell pores density was found by Tůmová et al. (2011) who revealed that higher pores number was observed in cages than on litter housing system.

Numerous studies have demonstrated a different incidence of cracked eggs in cages housing systems. Although, Vits et al. (2005) reported stronger eggshells from birds in enriched cages compared with conventional cages, Wall et al. (2002) observed a lower percentage of broken eggs collected from hens in conventional cages compared with enriched cages. These contrast results are presumably related to calcium metabolism, because the most commonly used indicators of Ca metabolism in layers are shell quality assessment parameters (Gordon and Roland, 1998). Neijat et al., (2011) indicated that enriched cages may provide better means of

utilizing Ca and P than in conventional cages. These results might be affected by higher feed consumption in enriched cages. Hence, giving attention to Ca and P feed content may improve the eggshell quality parameters in alternative housing systems.
Other factors contributing to the proportion of cracked eggs are cage design, egg savers, and nest floor material. Guesdon et al. (2006) explained that differences in egg breakage may be because of the influence of cage design elements, including the presence of perches (Abrahamsson and Tauson, 1998), rather than specific cage effects.

Not each genotype performs the same in certain housing system. Therefore, the interactions between the housing system and genotype has a great effect on eggshell quality characteristics. For instance, it was recommended by Singh et al. (2009) that the strain should be considered when using housing systems. Eggshell weight was affected by the interactions of housing and genotype in the study of Tůmová et al. (2011) which conducted on three housing system (cages, litter and enriched cages) and three laying hens genotype (ISA Brown, Bovans Brown and Moravia BSL). The authors found heavier eggshells in all genotypes on litter system than conventional cages and enriched cages. Leyendecker et al. (2001b) studied the interactions between genotype and housing system for eggshell thickness in an experiment of Lohmann LSL and Lohmann Brown housed in conventional cages, aviaries and intensive free-range system. They found thicker eggshells in the intensive free-range for both laying hens lines than conventional cages and aviaries. However, it might be assumed that, the interactions of housing system and genotype may play an important role on eggshell quality than the individual factors. Therefore, it is highly recommended to choose the genotype which suit with the type of housing systems which might reflect eggs with better eggshell characteristics.

Adequate feeding regime reflects a better eggshell quality especially feed with balanced mineral content of calcium and phosphorus. Calcium nutrition is a key element for eggshell quality, each eggshell contains up to 3 grams of calcium so the diet of hens must contain adequate amount of calcium in a form that can be utilized efficiently (Roberts, 2010). Because both excess and deficiency of calcium negatively affect the shell quality, the NRC (1994) estimated the Ca requirement of laying hens to be 32.5 g/kg diet at 100 g of feed intake per day. Moreover, the calcium source and particles may play an important role in improving eggshell quality. Lichovníková, (2007) observed higher eggshell weight, thickness and strength of laying hen eggs fed calcium with large limestone particles size compared with the fine one.

Phosphorus is the second main mineral in the eggshell; it does not only occur in vesicles in the shell cuticle but is incorporated at very low concentrations into the outer regions of the eggshell, P concentration then increases until eggshell termination, supporting the concept of the role of P in termination of eggshell formation (Cusack et al., 2003). Numerous studies have shown that eggshell quality is lowered by high dietary levels of available P the negative effect being significant when the dietary non-phytate phosphorus is higher than 0.35-0.4 % (Nys et al., 2001).

The importance of trace elements (copper, zinc, manganese) has been demonstrated in the changes of arrangement pattern of shell membrane fibers in relation to the structural composition of the eggshell. Certain dietary levels and sources of trace elements mainly Zn and Mn, influence the metabolic indices of the gastrointestinal tract, and can beneficially affect the eggshell mineralization process and eggshell quality. The absence of supplementation of these elements decreases eggshell weight but this is mainly due to an absence of manganese (Abdallah et al., 1994). Venglovska et al. (2014) observed positive effects of Mn on eggshell quality with beneficial importance from organic sources.

Vitamins such as vitamin D3 are necessary for calcium metabolism and must be included in the diet. Vitamin D3, the only form that is effective in birds, has a role in the control of calcium metabolism in the chicken, in particular in the intestinal absorption of calcium which is directly dependent on its active metabolite, 1.25 dihydroxy-cholecalciferol (Bar, 2008). A positive effect on shell quality has been reported in chickens in the late production stage (Koreleski and Swiatkiewicz, 2005). However, the challenge for the future will be to define sustainable feed systems for those which impact least on the environment while guaranteeing the quality of the final product by the time it reaches the consumer.

Environmental conditions within the laying hen house, and the system of management, are also crucial components which can change its feed consumption. This can lead to unpredictable changes in both egg production and quality. Higher environmental temperature reduces feed intake and limits the availability of blood calcium for egg shell formation. During exposure to warm environmental temperature, the hen reacts by increasing its rate of breathing (panting) in order to cool itself. This causes the lowering of CO_2 in the blood. It may also reduce the activity of carbonic anhydrase, enzyme which results in the formation of bicarbonate which contributes the carbonate to the eggshell (Balnave et al., 1989). Therefore, sodium bicarbonate supplementation during heat stress may improve egg shell quality (Altan et al., 2000). For many years, researchers have been investigating the effect of high

environmental temperature on eggshell quality of laying hens. High ambient temperature resulted in a significant reduction in eggshell weight (Roberts, 2004; Franco-Jimenez et al., 2007; Sahin et al., 2009), lower eggshell thickness and strength (Oguntunji and Alabi, 2010; Lin et al., 2004; Ebeid et al., 2012; Tůmová et al., 2014) and increased eggshell breakage (Lin et al., 2004). Therefore, during the hot weather period, it is important to focus on feeding essential nutrients mainly Ca, P and vitamin D_3. Moreover, it should be recognized that birds will tend to eat most during the cooler times of the day.

Lightning is an important external factor that influences the eggshell quality of laying hens. Regarding the use of artificial lighting for laying hens, the practice of management extends the day length, providing a suitable light regime for laying hens, which brings the benefits of opportunity to advance or delay the laying onset, influence on the improvement of eggshell quality. Er et al. (2007) found that eggshell quality of commercial layer eggs was statistically affected by monochromatic red, green, and blue light compared with incandescent lamps. The authors found that eggshell index, thickness and eggshell strength were significantly higher when green light was used relative to the other treatments.

A range of types of general stress can affect egg shell quality. High population densities were shown some time ago to increase the production of body-checked eggs (Roberts, 2010). Body-checked eggs are thought to result from contraction of the shell gland while the eggshell is in the early stages of formation. Stress can also induce delays in the timing of oviposition when hens retain their eggs and this can result in an increased incidence of white-banded and slab-sided eggs (Reynard and Savory, 1999). Moreover, eggshell quality deterioration associated with heat stress is a well-known phenomenon, there is three major factors contributing to heat stress, reproductive failure (fewer eggs), poor egg quality (soft shells or shell-less eggs), and impaired skeletal integrity of the hen (Sahin et al., 2007). Heat stress reduces feed intake and limits the availability of blood calcium for egg shell formation. It may also reduce the activity of carbonic anhydrase, an enzyme which results in the formation of bicarbonate which contributes the carbonate to the egg shell (Roberts, 2010).

2.5. Factors affecting egg cuticle deposition and egg microbial contamination

Under healthy breeding conditions, an egg's contents are generally sterile just after laying. However, they can be contaminated by a diversified microbiota containing food spoilage microorganisms and sometimes pathogenic bacteria. Eggs can be contaminated externally, on

the eggshell, and internally during development. The egg may therefore be a vector of bacteria causing foodborne illness in humans such as *Salmonella*.

Cuticle deposition is important for the prevention of micro-organisms penetration, which is frequent event in the absence of cuticle deposition. This feature is reserved by the antimicrobial substances such as lysozyme and ovo-transferrin deposited in eggshell cuticle (Rose-Martel et al., 2012; Mikšík et al., 2014). Additionally, Messens et al. (2007); De Reu et al. (2010); Bain et al. (2013) observed a high correlations between the absence of cuticle and bacterial penetration across the eggshell. Cuticle also is important to create a barrier which inhibits water movement across the shell and prevents dehydration of the egg interior components (Rose-Martel et al., 2012).

The deposition of eggshell cuticle is affected by wide range of factors. Samiullah and Roberts (2014) reported a significantly higher cuticle deposition in cages versus free-range eggs. On the other hand, Ketta and Tůmová (2018) did not find significant affect between cages and litter systems on eggshell cuticle. This contrast results might be explained by Kusuda et al. (2011) who concluded that the diversity in the structure of the cuticle layer may be linked to the environment of the nest, mainly humidity.

The safety of egg production depends on eggshell contamination and the penetration of microorganisms into the egg. Recently, a greater attention was given to the effect of housing system on egg hygiene as the non-cage systems may have consequences on egg safety represented in increasing the percentage of cracked and dirty egg.

Englmaierová et al. (2014) reported significant effect of housing systems on the total count of bacteria on the egg surface and the microbial contamination of *Enterococcus* and *Escherichia coli*. The lowest values for the total count of bacterial contamination were found in eggs from conventional cages (4.05 log colony-forming units (CFU)/egg) and enriched cages (3.98 log CFU/egg) while, the highest level of contamination was observed in eggs that were laid on litter (6.24 log CFU/egg). These findings are in agreement with De Reu et al. (2006a) who reported a significant higher average eggshell contamination by aerobic bacteria and the Gram-negative bacteria of eggs from alternative housing systems compared to conventional cages. Comparing egg contamination between enriched and conventional cages Wall et al. (2008) reported that the proportions of dirty eggs were 4.2 and 5.4% in enriched and conventional cages, respectively which means that in well-designed enriched cages it is possible to achieve similar results regarding proportions of dirty eggs as in conventional cages. Moreover, De Reu et al. (2006b) and Messens et al. (2007) proved that higher eggshell

contamination led to a greater possibility of microorganism penetration and egg content contamination, which may be related with a higher contamination of eggs in alternative housing systems.

Hens of different genotypes might differ in their percentage of cuticle deposition and consequently egg contamination. In their study to detect the effect of different laying hen genotypes on eggshell cuticle deposition, Ketta and Tůmová (2018) indicated that the laying hen genotype plays an important role in the deposition process. Higher cuticle coverage was in eggs produced by Lohmann Brown compared to Isa Brown and Hy-Line Silver Brown. Moreover, Samiullah and Roberts (2014) suggested that brown eggs have the ability to prevent bacterial penetration more than white eggs which might be related to higher cuticle deposition in brown eggs. Moreover, Berthelot et al. (1998) have shown that hen resistance to caecal colonization by *Salmonella* Enteritidis has a heritable genetic basis. Similarly, Sadeyen et al. (2006) comparing two lineages of hens have observed significant differences in the expression of several genes encoding proteins involved in the defense against colonization by Salmonella. Thus, hen selection may be an efficient way to improve resistance to colonization by *Salmonella*.

Age is a very important factor affecting the deposition of eggshell cuticle. For instance, it is well known that there is a gradual decline in the quality of the cuticle with hen age (Leleu et al., 2011). This observation was confirmed by Rodriguez-Navarro et al. (2013) who indicated that the eggs from end-of-lay hens generally have a very poor degree of cuticle coverage. However, there is scant information on how eggshell cuticle composition is affected hen age.
The age of the hen is also important to resist the bacterial contamination; the hen resistance to *Salmonella* generally increases with age. The reason could be connected with the development of a mature intestinal flora and an effective immune system (Suzuki, 1994). Samiullah et al. (2014) studied the effect of age (25, 35, 45, 55, 65, and 75 weeks) on eggshell microbial contamination, and reported significantly higher *enterococcus* contamination at the age of 75 compared to eggs produced by younger hens. However, De Reu et al. (2006b) found no influence of hen age on bacterial eggshell penetration and egg content contamination for eggs of 34, 46, 60, 69 and 74 weeks.

Research results are varies on the effect of egg washing on eggshell contamination and egg bacterial penetration. It is argued that egg washing decreases the level of eggshell contamination and, consequently, the level of internal and external egg contamination (Jones

et al., 2004). On the other hand, egg washing is considered to be responsible for weakening the external barriers of the egg, such as the cuticle, and for an increase in humidity (Favier et al., 2000; Samiullah et al., 2013).

3. Scientific Hypothesis and Objectives

3.1. Hypothesis

The eggshell quality traits play an important role concerning profitability as only eggs with an intact shell are considered for hatching or as table eggs. Therefore, if the eggshell quality parameters (mainly thickness and strength) are guaranteed, the industry could increase the number of eggs produced by each hen housed. The thickness of the eggshell as an important indicator for overall eggshell quality. Therefore, would eggs of different eggshell thickness affect other eggshell characteristics when hens housed in different housing systems? The hypothesis was also set to compare the affectivity of housing system and genotype and the interactions between them on eggshell quality parameters and cuticle deposition. Using constant genotype, would the interactions of housing system, age and storage time have effect on the eggshell quality and egg safety?

3.2. Objectives

Regarding to the importance of eggshell mentioned above, it is necessary to study which factors might affect its characteristics. Furthermore, studying the possible interactions between those factors.

The aim of the study was to investigate the relationship between eggshell quality parameters in two different housing systems. In addition, to study the interactions of housing system and genotype on eggshell quality and cuticle deposition. Finally, to evaluate the effect of the housing system, age and their possible interactions on eggshell quality, microbial contamination and the penetration of microorganisms during different storage time.

4. Materials and Methods

During the PhD study, four experiments were done. All the experiments were approved by the Ethics Committee of the Czech University of Life Sciences Prague and the Central Commission for Animal Welfare at the Ministry of Agriculture of the Czech Republic.

4.1. Experiment 1

The experiment was done at Czech University of Life sciences Prague, Faculty of Agrobiology Food and Natural Resources, Department of Animal Husbandry, Prague, Czech Republic.

The study was designed to determine the relationship between eggshell thickness and other eggshell measurements in eggs produced on litter and enriched cages. The eggshell quality parameters were evaluated in 200 laying hens of ISA Brown at the age of 40-42 weeks. Laying hens were housed in enriched cages (100 hens, 750 cm^2/hen, 10 hens/cage) and on littered pens with wood shavings (100 hens, 9 hens/m^2, 10 hens/pen). The eggs were split into three categories differed in its thickness: the first category (thin shells; 0.28 - 0.30 mm, 377 eggs from enriched cages and 312 eggs from litter system), the second category (medium shells; 0.33-0.36 mm, 497 eggs from enriched cages and 291 eggs from litter system) and the third category (thick shells; 0.39-0.41 mm, 405 eggs from enriched cages and 424 eggs from litter system). Laying hens in both housing systems were fed identical commercial feed mixture with 15.37% crude protein, 11.58 MJ of metabolizable energy, 3.48% calcium and 0.56% of total phosphorous. Feed and water were supplied ad libitum. The daily photoperiod consisted of 14 h light, with an intensity of 10 lx at bird head level. The environmental conditions were kept according to the method described by Skřivan et al. (2015).

Eggs were analyzed every week three days in a row. The eggshell parameters were measured including: egg weight, length and width of the egg, eggshell strength, eggshell thickness, eggshell percentage, egg surface area and eggshell index. The relationship between eggshell parameters was evaluated by estimating Pearson's correlations coefficient.

4.2. Experiment 2

The experiment was done at Czech University of Life sciences Prague, Faculty of Agrobiology Food and Natural Resources, Department of Animal Husbandry, Prague, Czech Republic.

The study investigated the differences in the eggshell quality and the tibia measurements between Lohmann White and Czech Hens housed in conventional cages and on litter system.

Total number of 123 laying hens of Lohmann White and pure breed Czech Hen were housed in conventional cages Eurovent (72 hens, 550 cm^2/hen, 3 hens in a cage, 12 cages for genotype) and in six littered pens (60 hens, 7 hens/m^2, 10 hens/pen and 3 pens for each genotype). The experiment was carried out in the second half of laying cycle. Laying hens in both housing systems were fed commercial type of feed mixtures. The daily photoperiod consisted of 15 h light and 9 h darkness. Eggs for the egg shell quality assessment were collected in two weeks interval, two days in row, all eggs laid from each cage or litter pen and there were analyzed 300 eggs of Lohmann and 150 eggs of Czech Hen. Eggs were weighed, and the shell strength was determined by the shell-breaking method using a QC-SPA device (TSS York, UK). Eggshell weight was determined after drying. Eggshell thickness was evaluated by QCT shell thickness micrometer (TSS York, UK). Eggshell proportion was calculated from dried eggshell weight and egg weight.

Tibia characteristics were determined in 48 hens, 12 birds per a group, at 50 weeks of age. After slaughtering, both tibias were completely removed from the carcass. Weight, strength were measured in the right tibia. Tibia strength was measured by QC-SPA device (TSS York, UK) and thickness by micrometer QCT (TSS York, UK). Tibia Ca content was analyzed in the left tibia after ashing at 550 ^oC overnight using the method of AOAC 965.17 based on vanad-molybden reagent and spectrophotometry analysis on Solaar M6 apparatus (TJA Solutions, Cambridge, UK).

4.3. Experiment 3

The experiment was done in cooperation between the Central Institute for Supervising and Testing in Agriculture Ústrašice, and Czech University of Life sciences Prague, Faculty of Agrobiology Food and Natural Resources, Department of Animal Husbandry, Prague, Czech Republic.

The aim of the study was to compare the eggshell characteristics and cuticle deposition of Lohmann Brown, Hy-Line Silver Brown and Isa Brown housed in two different housing systems. The experiment was conducted on Lohmann Brown, Hy-Line Silver Brown and Isa Brown laying hens at the age of 40-56 weeks. Laying hens were housed in enriched cages (100 hens, 750 cm^2/hen, 10 hens/cage) and in littered pens (100 hens, 9 hens/m^2, 10 hens/pen). Laying hens in both housing systems were fed identical commercial feed mixture with 15.37% crude protein, 11.58 MJ of metabolizable energy, 3.48% calcium and 0.56% of total phosphorous. Feed and water were supplied *ad libitum*. The daily photoperiod consisted of 14 h light, with an intensity of 10 lx at bird head level. During the experiment, eggs were

collected in four weeks interval to be 660 eggs in total (20 eggs/ genotype/ housing system) and divided into two groups; 330 eggs were used for analysing of eggshell quality characteristics and the other 330 eggs were used to estimate cuticle deposition.

Freshly laid 330 eggs were used for eggshell quality assessments. The egg weight, length, width, shape index, strength, thickness, eggshell weight, percentage and egg surface area were evaluated.

The total number of 330 eggs were used for cuticle estimation by a method of Roberts et al. (2013).

The recorded average of L*, a*, and b* values, before and after staining was used to calculate ΔE^*_{ab}. $\Delta E^*_{ab} = \sqrt{[(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2]}$

A higher ΔE^*_{ab} denotes a higher staining affinity and hence more cuticle coverage (Leleu et al. 2011).

4.4. Experiment 4

The experiment was done in cooperation between Institute of Animal Science, Prague Uhříněves, Czech Republic, and Czech University of Life sciences Prague, Faculty of Agrobiology Food and Natural Resources, Department of Animal Husbandry, Prague, Czech Republic.

The study was oriented to evaluate the effect of housing system, and age on eggshell quality, microbial contamination and micro-organisms penetration into the eggs of Isa Brown. The experiment was conducted with ISA Brown hens. Laying hens were housed in enriched cages (60 hens, 10 hens per cage, 750 cm^2 per hen) and in free-range (60 hens, 9 hens per m^2) environments. The laying hens in the free-range environment were placed in one deep-litter pen with wood shavings and with access to run. The daily photoperiod consisted of 15 h of light and 9 h of darkness. Laying hens were fed identical commercial feed mixtures N1 (with 18.7% crude protein and 11.5 MJ of metabolizable energy) from 20 to 40 weeks of age and N2 (with 15.3% crude protein and 11.4 of metabolizable energy) from 41 weeks of age. Feed and water were supplied *ad libitum*. The microclimate conditions were in accordance with the laying hen´s requirements (Skřivan et al., 2015).

Eggs were collected for three consecutive days during the 26^{th} and 51^{st} week to determine egg weight, eggshell quality and pores density.

Microbial contamination analyses were done in eggs also collected during the 26^{th} and 51^{st} week of age, from different housing system and age. The microbial analysis of the eggshell surface and egg content was performed with fresh eggs and eggs stored at 2, 7, 14 and 21

days. The numbers of *Escherichia coli* (EC), *Enterococcus* (ENT) and the total number of microorganisms (TNM) were recorded.

The results of the experiments were evaluated with SAS program (SAS 9.4) using the GLM procedure. More detailed materials and methods are described below in the publications sections (chapter 5).

5. Publications

Ketta M., Tůmová E. (2018a): Relationship between eggshell thickness and other eggshell measurements in eggs from litter and cages. Italian Journal of Animal Science, 17, (1): 234-239. Doi: 10.1080/1828051X.2017.1344935

Ketta M., Tůmová E. (2014): Differences in the eggshell quality and tibia strength in Lohmann White and Czech Hen housed in cages and on litter. Acta Fytotechnica et Zootechnica, 17, (3): 75–78. DOI: 10.15414/afz.2014.17.03.75–78

Ketta M., Tůmová E. (2018b): Eggshell characteristics and cuticle deposition in three laying hen genotypes housed in enriched cages and on litter. Czech Journal of Animal Science, 63, (1): 11-16. Doi: 10.17221/75/2017-CJAS

Vlčková J., Tůmová E., **Ketta M**., Englmaierová M., Chodová D. (2018): Effect of housing system and age of laying hens on eggshell quality, microbial contamination, and penetration of microorganisms into eggs. Czech Journal of Animal Science, 63, (2): 51-60. Doi: 10.17221/77/2017-CJAS

ITALIAN JOURNAL OF ANIMAL SCIENCE, 2017
https://doi.org/10.1080/1828051X.2017.1344935

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Relationship between eggshell thickness and other eggshell measurements in eggs from litter and cages

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ABSTRACT

The objective of the present study was to determine the relationship between eggshell thickness and other eggshell characteristics in eggs produced in litter housing system and enriched cages. Eggs were collected from 200 birds of ISA Brown genotype at 40–42 weeks of age. Half of the birds were housed in enriched cages (750 cm^2/hen, 10 hens/cage) and the other half were housed in littered pens (9 hens/m^2, 10 hens/pen). Eggs in each housing system were split into three categories varying in shell thickness: the first category (thin shells 0.28–0.30 mm), the second category (medium shells 0.33–0.36 mm) and the third category (thick shells 0.39–0.41 mm). Results indicated that eggshell parameters differ significantly according to eggshell thickness. Significant interaction of shell category and housing system were observed in eggshell strength. As expected, the eggshell strength was increased with eggshells becoming thicker. Moreover, eggs with the thickest shells from enriched cages had significantly stronger shells than those from litter system. Eggshell weight was significantly increased in the thick eggshell category being higher in enriched cages (7.23 g) than in litter system (5.14 g). The Pearson's correlation coefficients showed a positive correlation between eggshell parameters and eggshell thickness in both housing systems. Moreover, the correlation between eggshell thickness and eggshell strength was higher on litter (0.64, $p < 0.001$) in comparison with enriched cages (0.48, $p < 0.001$). Results of the present study indicated that in thin shells, housing system plays an important role in determining the eggshell strength.

ARTICLE HISTORY
Received 18 January 2017
Revised 21 March 2017
Accepted 23 May 2017

KEYWORDS
Eggshell quality; eggshell strength; eggshell thickness; housing system

Introduction

The eggshell of laying hens still remaining one of the greatest interest of researchers, regarding to the economic losses of cracked and damaged eggs which accounts for 6 to 8% of total egg production (Hamilton et al. 1979); it also provides a pass way for micro-organisms penetration into the eggs. Therefore, improving overall eggshell quality would have a significant economic impact on egg production industry. The function of the eggshell is maintained by its structure as a complex of bio-ceramic material with 95% calcium carbonate (Nys et al. 1991).

Eggshell quality traits play an important role because only eggs with an intact shell are considered for hatching or as table eggs. Therefore, if the eggshell quality is guaranteed, the egg industry could increase the number of eggs produced by each hen housed. Eggshell thickness play a major role of these parameters of the eggshell. However, there is no direct effect of this parameters on the other eggshell properties.

Eggshell quality parameters might differ between housing systems (Tůmová and Ebeid 2005). Lichovníková and Zeman (2008) reported that heavier eggshells were produced in conventional cages compared to floor system and enriched cages. In contrast, the heaviest eggshells in litter system in comparison with conventional and enriched cages were observed by (Tůmová et al. 2011). Pavlovski et al. (2001) compared the effect of litter, free-range and cages housing systems on eggshell thickness and obtained thicker shells on litter eggs compared to free-range. Leyendecker et al. (2001) found that, lower eggshell thickness was obtained in eggs produced in cages while free-range eggs had the highest values of traits. Moreover, several studies reported that shell thickness was lower in eggs from cages than in litter housing

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system (Hidalgo et al. 2008; Tůmová et al. 2011; Ledvinka et al. 2012). Similarly, ambiguous results in different housing systems were observed for eggshell strength. Tůmová et al. (2011) observed the highest eggshell strength in cages system (4744 g/cm^2) compared to litter system (4651 g/cm^2). Similarly, Ledvinka et al. (2012) and Englmaierová et al. (2014) found stronger shells in cages compared to litter system. These results indicated that eggshell thickness and strength do not have the same trends in each housing system. Therefore, it is important to compare the relationship between eggshell quality characteristics according to housing system.

The differences in the physical variables like eggshell weight and thickness could explain nearly 60% of the eggshell strength variation (Frank et al. 1965). Several recent articles paid more attention to detect the relationship between shell thickness and strength. Yan et al. (2014) studied the effect of uniformity of eggshell thickness (a new parameter to evaluate eggshell quality, it is defined as the reciprocal of coefficient of variation of eggshell thickness from multiple positions) on eggshell quality and reported a positive correlation between shell thickness and breaking strength ($r = 0.319$) and static stiffness ($r = 0.425$), while they detected negative correlation with fracture toughness ($r = 0.472$). They also indicated that, eggs with thin but more uniform eggshell were stronger than those with thick but less uniform eggshell. Moreover, Kibala et al. (2015) reported a higher genetic correlations (0.8) between eggshell strength and its thickness making shell thickness as a selection index candidate element. However, thicker eggshells do not guarantee stiffer or stronger eggs (Bain 2005). Tůmová et al. (2011) and Ledvinka et al. (2012) indicated that, although shell thickness was lower in eggs produced in cages, eggshell strength was higher and this effect might be related to the ultra-structural features of the shells in cages eggs which presumably support the eggshell strength. Nevertheless, it might be assumed that housing system affect eggshell microstructure resulting different eggshell thickness and strength. Also, housing system had a significant effect on pores density (Ketta and Tůmová 2016).

The previous studies were conducted on one housing system and there is a question, whether housing systems affect eggshell characteristics when eggs differ in thickness? Therefore, the objective of the present study was to determine the relationship between eggshell thickness and other eggshell measurements in eggs produced in litter housing system and enriched cages.

Materials and methods

Experimental design and diets

Eggshell quality parameters were evaluated in an experiment with 200 laying hens of ISA Brown at the age of 40–42 weeks. Laying hens were housed in enriched cages (100 hens, 750 cm^2/hen, 10 hens/cage) and in littered pens with wood shavings (100 hens, 9 hens/m^2, 10 hens/pen).

According to eggshell thickness, eggs were split into three categories: the first category (thin shells; 0.28–0.30 mm, 377 eggs from enriched cages and 312 eggs from litter system), the second category (medium shells; 0.33–0.36 mm, 497 eggs from enriched cages and 291 eggs from litter system) and the third category (thick shells; 0.39–0.41 mm, 405 eggs from enriched cages and 424 eggs from litter system).

Laying hens in both housing systems were fed identical commercial feed mixture with 15.37% crude protein, 11.58 MJ of metabolisable energy, 3.48% calcium and 0.56% of total phosphorous. Feed and water were supplied *ad libitum*. The daily photoperiod consisted of 14 h light, with an intensity of 10 lx at bird head level. The environmental conditions were kept according to the method described by Skrivan et al. (2015).

Eggshell quality assessments

Eggs were analysed every week three days in a row, and individually weighed, length and width of each egg were measured for egg shape index calculation (width/length × 100). Eggshell strength was determined by the shell-breaking method using a QC-SPA device (TSS, England). After the eggs were broken, eggshell thickness was measured with a QCT shell thickness micrometer (TSS, England) at the equatorial area after removal of shell membranes. Eggshell weight was determined after drying according to (Englmaierová et al. 2015) and the eggshell percentage was calculated. The surface area of each egg was determined using the equation reported by Thompson et al. (1985): Egg surface area $= 4.67 \times (\text{egg weight})^{2/3}$. Eggshell index was calculated according to the followed equation: Eggshell index = (shell weight/shell surface) × 100 (Ahmed et al. 2005).

Statistical analysis

Data were statistically analysed using two-way analysis of variance (housing × shell thickness) using GLM procedure of SAS (SAS 2003). The relationship between eggshell parameters was evaluated by estimating Pearson's correlation coefficient.

Results

Interactions between shell thickness and housing system

Significant interaction of eggshell thickness category and housing system was detected for eggshell strength ($p < .05$), eggshell weight ($p < .05$), shell percentage ($p < .001$) and eggshell index ($p < .001$, Table 1). Egg weight was significantly affected by shell thickness category ($p < .001$), eggs become heavier with increasing shell thickness. In enriched cages, eggs were significantly ($p < .05$) heavier than in litter system. Eggshell strength were affected by the interaction between eggshell thickness category and housing system ($p < .034$). Eggshell strength did not differ according to housing system in the thick and medium category, however, in the thin category, eggs from enriched cages had significantly stronger shells in comparison with litter system. Also eggshell thickness category affected shell strength ($p < .001$), whereas shells being stronger in the thick category. Also, eggshell weight was significantly affected by the interaction of the shell thickness category and housing system. The highest eggshells weight were observed in thick shells category in enriched cages and the lightest eggshells were detected in thin shells category in litter system. Moreover, data showed that eggshell weight significantly differed in enriched cages and in litter system in the thick and the thin shells categories, whereas in the medium shell category was not affected. Eggshell weight was increased with eggshell thickness category ($p < .001$). Similar trends of the significant interaction of the eggshell thickness category and housing system were observed in eggshell percentage ($p < .001$) and eggshell index ($p < .001$). In contrast with the shell weight, eggshell percentage and eggshell index were not significantly ($p < .05$) affected by housing system. However, the interaction showed a higher values of traits in cage system compared to litter mainly in the thin category. Eggshell surface significantly increased with the shell thickness category.

Pearson's correlation coefficients of eggshell parameters

The Pearson's correlation coefficients of eggshell parameters of eggs produced in enriched cages (Table 2) and in litter system (Table 3) indicated positive correlations among eggshell thickness and the other eggshell parameters. Also, in other eggshell measurements, higher correlations were observed in litter system than in enriched cages except the

Table 1. The effect of eggshell categories and housing systems on eggshell parameters.

Eggshell T. category	Housing system	Egg weight (g)	Egg shape index (%)	Eggshell strength (g/cm^2)	Eggshell thickness (mm)	Eggshell weight (g)	Eggshell percentage (%)	eggshell surface area (cm^2)	Eggshell index (g/100cm^2)
Thin	Cage	64.20	76.27	3812^{e}	0.290	5.46^{d}	10.61^{c}	74.83	9.09^{c}
	Litter	63.62	76.49	3339^{d}	0.286	5.14^{e}	10.07^{d}	74.31	8.57^{d}
Medium	Cage	66.40	76.79	4686^{b}	0.356	6.40^{c}	11.57^{ab}	76.50	10.01^{ab}
	Litter	64.83	76.98	4590^{b}	0.356	6.30^{c}	11.58^{ab}	75.32	9.95^{ab}
Thick	Cage	67.60	77.23	5188^{a}	0.403	7.23^{a}	12.60^{a}	76.86	10.94^{a}
	Litter	66.82	77.09	5183^{a}	0.402	7.08^{b}	12.22^{b}	77.44	10.64^{a}
Root Mean Square Error		5.56	3.04	702	0.012	0.47	0.74	4.20	0.54
Shell thickness		0.001	0.141	0.001	0.001	0.001	0.001	0.001	0.001
Housing system		0.050	0.739	0.003	0.150	0.036	0.436	0.053	0.569
Shell thickness × Housing		0.561	0.793	0.034	0.317	0.003	0.001	0.611	0.001

^{a,b,c,d,e}statistically significant differences ($p \leq .05$) within columns are indicated by different superscripts.

Table 2. Pearson's correlation coefficients between eggshell parameters of eggs produced in cages ($n < 1279$).

	Egg weight	Eggshell strength	Eggshell thickness	Eggshell weight	Egg shape index	Eggshell percentage	Eggshell surface area
Eggshell strength	−0.12*						
Eggshell thickness	0.15**	0.48***					
Eggshell weight	0.64***	0.35***	0.70***				
Egg shape index	0.12*	0.13**	0.05	0.07			
Eggshell percentage	−0.45***	0.49***	0.51***	0.31***	−0.08		
Eggshell surface area	0.99***	−0.11*	0.15**	0.64***	0.12*	−0.45***	
Eggshell index	−0.06	0.50***	0.64***	0.64***	−0.04	0.91***	−0.05

*$p \leq .05$.
**$p \leq .01$.
***$p \leq .001$.

Table 3. Pearson's correlation coefficients between eggshell parameters of eggs produced on Litter ($n < 1027$).

	Egg weight	Eggshell strength	Eggshell thickness	Eggshell weight	Egg shape index	Eggshell percentage	Eggshell surface area
Eggshell strength	0.16**						
Eggshell thickness	0.20***	0.64***					
Eggshell weight	0.56***	0.62***	0.85***				
Egg shape index	0.06	0.18**	0.05	0.03			
Eggshell percentage	−0.21***	0.53***	0.74***	0.65***	−0.04		
Eggshell surface area	0.99***	0.16**	0.21***	0.56***	0.05	−0.21***	
Eggshell index	0.09*	0.60***	0.82***	0.83***	−0.02	0.95***	0.09*

*$p \leq .05$.
**$p \leq .01$.
***$p \leq .001$.

eggshell surface area. In enriched cages, negative correlation ($p < .05$) between eggshell strength and eggshell surface area was detected, while, in litter system positive correlation ($p < .01$) was obtained. A negative correlation ($p < .001$) was detected between eggshell percentage and eggshell surface area with higher values in litter system than in cages.

Discussion

The obtained results revealed that the different shells thickness categories significantly affected the other eggshell quality characteristics. The egg weight significantly increased when the eggs become thicker. The increasing in egg weight might be related to higher eggshell weight which also increased with the eggshell thickness category. This relationship was estimated by correlations between egg weight and eggshell weight (0.64 in enriched cages and 0.56 in litter system). Lower correlations in litter system might be assumed to be affected by significantly lower egg weight in litter system. Results of the present study indicated a significant interaction of shell thickness category and housing system for eggshell strength. The eggshell strength significantly increased as the eggshells become thicker; with different values between eggs produced in enriched cages and in litter system especially in the thin shell category, while shell strength did not differ between litter and enriched cages in the medium and thick shell categories. These results are in agreement with Kibala et al. (2015) who reported a positive genetic correlations between eggshell strength and its thickness. On the other hand, Tatara et al. (2016) revealed a negative correlation between eggshell thickness and eggshell strength, indicating that mechanical endurance of the eggshell is not simply affected by its thickness but other factors such as mineral density, mineral content and spatial micro architectural arrangement contribute to this characteristic. Results of the present study indicated a significantly positive correlation between egg shape index and eggshell strength in both housing systems. The larger, and rounder eggshells have the higher resistance to breaking forces. These results are in agreement with the findings of Anderson et al. (2004) and Blanco et al. (2014) who reported positive correlation between eggshell strength and egg shape index. Therefore, it is necessary to monitor egg shape to maintain an optimal form for stronger eggshells.

The interaction of shell thickness category and housing system was also observed for eggshell percentage. The thin and thick shells categories showed big differences between enriched cages and litter system for eggshell percentage, while the medium shell category did not differ. These results might be related to the uniformity of eggshell thickness as Yan et al. (2014) reported that eggs with thin but more uniform eggshell showed better shell measurements than those with thick but less uniform eggshells. A higher negative correlation between eggshell percentage and

egg weight was found in litter system compared to enriched cages. While, the correlation between eggshell thickness; eggshell weight and eggshell percentage were highly positive in litter system. The eggshell index was similar to eggshell percentage being significantly different between enriched cages and litter systems in the thin and thick shells category only with higher values in enriched cages than in litter system. Also the correlation between eggshell thickness; eggshell weight and eggshell index were positively higher in litter system than in enriched cages. The different results between both housing systems might be assumed as the greater chance of eggshell contamination exists in litter system compared to enriched cages. Eggshell index is related to shells crystal size and lower values indicate larger crystals which causes lower eggshell strength (Ahmed et al. 2005). Based on the lower values of the eggshell index in the thin category, it might be assumed that the thin shells mainly in litter system are created from larger crystals which result to lower eggshell strength. It seems that eggshell structure in the thin shells plays more important role than in eggs with thick shells mainly in litter housing system.

Conclusions

In conclusion, the current study results investigated the important relationship between eggshell thickness and other eggshell parameters. As expected, the eggshell strength increased with eggshells becoming thicker. Moreover, eggs with the thickest shells from enriched cages had significantly stronger shells than those from litter system. These results indicated that in the thin shell thickness, housing system plays an important role in the relationship to strength which might be related to the crystals size and orientation as the major determinant of shell thickness and strength. More attention should be paid to the egg shape to ensure better shell quality characteristics.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

The study was supported by the Ministry of Agriculture of the Czech Republic, Project NAAR NO. QJ1310002.

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Acta fytotechn. zootechn., 17, 2014(3): 75–78

Original Paper

Differences in the eggshell quality and tibia strength in Lohmann White and Czech Hen housed in cages and on litter

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This study investigated the differences in the eggshell quality and the tibia measurements between Lohmann White and Czech Hens housed in conventional cages and on litter system. The significant interactions between genotype and housing system were detected for the egg weight; the significantly heaviest eggs ($P \leq 0.001$) were in Lohmann White from cages and the lightest weight in Czech Hen in both housing systems. There was also significant interaction of genotype and housing system in the shell thickness, with the significantly thickest eggshells ($P \leq 0.003$) in Lohmann White from litter system (0.357 mm) and the thinnest in Czech hen housed in cages (0.310 mm). From tibia strength characteristics only the tibia strength was affected by interactions ($P \leq 0.004$), the tibia strength was higher in Czech Hen on litter system (498 N) than in conventional cages (318 N). Serum calcium concentration and pore density were not significantly influenced by the interaction of genotype and housing system. The results indicated that genotypes can have a different reaction in the eggshell quality depending on housing system, and these interactions can be more important than individual factors.

Keywords: eggshell quality, genotype, housing system, serum Ca, tibia strength

1. Introduction

Eggshell quality is one of the most important problems facing poultry industry; it economically influences egg production and hatchability. The eggshell quality is often expressed through its weight, percentage, thickness and strength. Composition of the eggshell and its characteristics are affected by many factors from which housing system and genotype are very important. Several studies were done in order to evaluate the effect of housing systems on eggshell quality including cages and litter systems, and to indicate which housing system is more effective for better eggshell quality. Pištěková et al. (2006), Zemková et al. (2007) and Singh et al. (2009) detected heavier eggs on litter, whereas Moorthy et al. (2000), Leyendecker et al. (2001a); Lichovníková and Zeman (2008) and Tůmová et al. (2011) found heavier eggs in cages. Hidalgo et al. (2008) showed the effect of housing on the eggshell thickness and the strength, they stated that the shell thickness was the lowest in eggs produced in cages, while barn eggs presented the highest values. On the other hand, Pištěková et al. (2006) suggested that difference in the shell strength in eggs from deep litter system and in eggs from cage system was not found statistically significant. Pores density was higher in cage system than on litter (Tůmová et al., 2011). Eggshell parameters can be related to serum calcium concentration, because it is the major structural element in the eggshell and large amounts of Ca are required to synthesize the shell. Řezáč et al. (2000) reported that the highest serum Ca levels in laying hens producing eggs with damaged shells, similar results were found by Pavlík et al. (2009) who reported that increased serum Ca level was associated with decreasing the eggshell strength and the thickness.

Tibia breaking strength is an important welfare problem for laying hens. Leyendecker et al. (2001a) suggested that the eggshell stability and the thickness seem to be negatively correlated with the bone strength. Several studies have shown a higher incidence of bone fragility in caged laying hens compared to hens kept in alternative housing system. Leyendecker et al. (2005) reported that, the weakness of the bones of hens kept in conventional cages is estimated to be mainly due to the limited opportunity to exercise.

Not only housing system affects eggshell quality but also genotype has a great effect on eggshell characteristics (Tůmová et al., 2007; Zita et al., 2009; Ledvinka et al., 2011). Tůmová et al., (2007) confirmed the effect of genotype on the eggshell weight which were higher in eggs of Plymouth Rock strain than Blue strain.

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Faculty of Agriobiology and Food Resources, 2014
http://www.fapz.uniag.sk/
DOI: 10.15414/afz.2014.17.03.75–78

Ledvinka et al. (2011) observed the significant effect of genotype on the eggshell thickness and the eggshell strength. On the other hand, Leyendecker et al. (2001b) found a thinner eggshell in brown eggs compared to the white ones. Basmacioglu and Ergul (2005) did not report a significant effect of the genotype on the shell strength and the thickness. Moreover, non-significant differences in the shell strength were determined by Tůmová et al. (2007) in variable brown strains.

Eggshell quality is also influenced by interaction between housing and genotype. Leyendecker et al. (2001b) reported genotype and housing system interactions on eggshell parameters. Vits et al. (2005) pointed out that eggshell quality characteristics were lower in enriched cages than in conventional cages, and that Lohmann Brown hens showed better results compared to Lohmann LSL. Singh et al. (2009) suggested that strain should be considered when using alternative housing systems.

The present study was conducted to investigate the differences in the eggshell parameters and the tibia strength in Lohmann White and Czech Hen housed in cages and on litter.

2. Material and methods

In the experiment with 123 laying hens of Lohmann White and pure breed Czech Hen, birds were housed in conventional cages Eurovent (72 hens, 550 cm^2 hen, 3 hens in a cage, 12 cages for genotype) and in six littered pens (60 hens, 7 hens per m^2, 10 hens per pen and 3 pens for each genotype). The experiment was carried out in the second half of the laying cycle. Laying hens in both housing systems were fed commercial type of feed mixtures. The daily photoperiod consisted of 15 h light and 9 h darkness. Eggs for the egg shell quality assessment were collected in two weeks interval, two days in row, all eggs laid from each cage or litter pen and there were analyzed 300 eggs of Lohmann and 150 eggs of Czech Hen. Eggs were weighed, and the shell strength was determined by the shell-breaking method using a QC-SPA device (TSS York, UK). Egg shell weight was determined after drying. Egg shell thickness was evaluated by QCT shell thickness micrometer (TSS York, UK). Egg shell proportion was calculated from egg shell weight, which was determined after drying, and egg weight.

Tibia characteristics were determined in 48 hens, 12 birds per a group, at 50 weeks of age. After slaughtering, both tibias were completely removed from the carcass. Weight, strength were measured in the right tibia. Tibia strength was measured by QC-SPA device (TSS York, UK) and thickness by micrometer QCT (TSS York, UK). Tibia Ca content was analysed in the left tibia after ashing at 550 °C overnight using a method AOAC 965.17 based on vanad-molybden reagent and spectrophotometry analysis on Solaar M6 apparatus (TJA Solutions, Cambridge, UK).

Egg shell quality data and tibia measurements were evaluated by two-way (housing, genotype) analysis of variance using the GLM procedure of SAS (SAS Institute Inc., Cary, Nc, 2003).

3. Results and discussion

In our study, significant interaction between housing system and genotype were detected. The significant interaction of housing system and genotype revealed that egg weight (Table 1) was higher in Lohmann White than Czech Hen, which is in accordance with results of Tůmová et al. (2009), Singh et al. (2009) and Ledvinka et al. (2012) who found interaction between housing system and genotype for the egg weight. Lohmann White hens produced significantly heavier eggs in cages than on litter; however, in Czech Hen the egg weight was not affected by housing system. Results of Lohmann White in the egg weight are in agreement with Moorthy et al. (2000), Leyendecker et al. (2001a), and Jenderal et al. (2004), who found heavier eggs in cages. On the other hand, Tůmová and Ebeid (2005), Pištěková et al. (2006), Zemková et al. (2007), and Singh et al. (2009) that detected heavier eggs on litter.

The eggshell percentage was not affected by evaluated factors. The eggshell thickness was the significantly highest ($P \leq 0.003$) in Lohmann White on litter; Lohmann White produced thicker shells than Czech

Table 1 Mean of eggshell parameters from Lohmann White and Czech Hen housed in cages and litter

	Lohmann White		Czech Hen		RMSE	Significance Genotype × Housing
	cages	litter	cages	litter		
Egg weight in g	61.18[a]	60.05[b]	49.04[c]	49.19[c]	6.134	0.001
Eggshell percentage in %	12.23	12.20	11.33	11.55	0.903	0.101
Eggshell thickness in mm	0.352[b]	0.357[a]	0.310[d]	0.322[c]	0.028	0.003
Eggshell strength in g / kg	4358	4384	4157	4186	903.6	0.696

[a, b, c, d] statistically significant differences ($P \leq 0.05$) within columns are indicated by different superscripts
RMSE-root mean square error

Table 2 Mean of pore numbers from Lohmann White and Czech Hen housed in cages and litter

	Lohmann White		Czech Hen		Significance Genotype × Housing
	cages	litter	cages	litter	
Blunt end	112.4	110.0	105.4	103.5	NS
Sharp end	41.8	19.6	44.3	24.4	NS
Equatorial	102.9	99.8	96.6	98.6	NS

Table 3 Mean of tibia parameters and serum Ca concentration from Lohmann White and Czech Hen housed in cages and litter

	Lohmann White		Czech Hen		Significance Genotype × Housing
	cages	litter	cages	litter	
Tibia weight in g	4.68	5.18	5.18	5.58	NS
Tibia strength in N	318d	460c	477b	498a	0.004
Serum Ca in mm / l	9.56	10.12	2.53	4.96	NS

[a, b, c, d] statistically significant differences ($P \leq 0.05$) within columns are indicated by different superscripts

Hen. The eggshell thickness was higher on litter system than in cages; however, Hidalgo et al. (2008) showed that shell thickness was the lowest in eggs produced in cages, while free-range and barn eggs presented the highest values. Non-significant interactions were detected for the eggshell strength. On the other hand, Tůmová et al. (2011) found significant interactions of housing and genotype in brown-egg hybrid kept in conventional cages and on litter.

The eggshell quality can be also characterized by pore density. There were no significant interactions between genotype and housing system on eggshell pore density (Table 2). On the other hand, we found numerically higher pore density in cages than on litter, especially in the shell sharp end. Similar results were reported by Tůmová et al. (2011), who detected a higher pore density on the sharp end and in the equatorial area in eggs from hens from litter.

The significant interactions ($P \leq 0.004$) in the tibia strength were observed between housing system and genotype (Table 3) which is in agreement with results of Vits et al. (2005). Tibia strength in Czech Hens was stronger in both housing systems than those of Lohmann White.

Moreover the effect of housing system on the tibia breaking strength was found. The tibia strength was higher on litter system than in conventional cages which is in agreement with Newman and Leeson (1998), Leyendecker et al. (2001c), on the other hand Vits et al. (2005) did not detect difference in tibia strength among the housing systems. Leyendecker et al. (2005) reported that, the weakness of the bones of hens kept in conventional cages is estimated to be mainly due to the limited opportunity to exercise. Serum Ca concentration also did not significantly affected by interaction between housing system and genotype; however, numerically higher concentration was in Lohmann White.

4. Conclusions

The results of the present investigation showed significant interactions between genotype and housing systems on the egg weight, the eggshell thickness and the tibia strength. The results indicated that genotypes can have a different reaction in the eggshell quality depending on housing system, and these interactions can be more important than individual factors.

5. Acknowledgements

The study was supported by project of NAAR number QI 101A164 and internal project of FANFR.

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DOI: http://dx.doi.org/10.2754/avb200978010085.

doi: 10.17221/75/2017-CJAS

Eggshell Characteristics and Cuticle Deposition in Three Laying Hen Genotypes Housed in Enriched Cages and on Litter

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ABSTRACT

Ketta M., Tumova E. (2018): **Eggshell characteristics and cuticle deposition in three laying hen genotypes housed in enriched cages and on litter**. Czech J. Anim. Sci., 63, 11–16.

The objective of the present study was to compare the eggshell characteristics and cuticle deposition of Lohmann Brown, Hy-Line Silver Brown, and Isa Brown layers kept in two different housing systems. The three laying hen genotypes were housed in enriched cages (100 hens, 750 cm^2/hen, 10 hens/cage) and in littered pens (100 hens, 9 hens/m^2, 10 hens/pen). The experiment was carried out in weeks 40–56 of hens age. Non-significant interactions of genotype and housing system for eggshell quality parameters and cuticle deposition were detected in this study. Egg weight was significantly affected by genotype ($P \leq 0.001$) and housing system ($P \leq 0.043$). The heaviest eggs were laid by Lohmann Brown, while the lightest eggs were produced by Hy-Line Silver Brown. Eggshell strength was not affected by genotype and housing system, however, genotype had a significant effect on eggshell thickness ($P \leq 0.033$). Isa Brown eggs had thicker eggshells compared to Lohmann Brown and Hy-Line Silver Brown. However, a non-significant effect of housing system on eggshell thickness was observed. Eggshell percentage was significantly affected by both genotype and housing system. Genotype of laying hens had a significant effect on cuticle deposition; significantly higher cuticle deposition was observed in Lohmann Brown eggs ($P \leq 0.001$). It could be concluded that genotype had a significant effect on eggshell quality parameters and cuticle deposition. However, the housing system effect was less important in these characteristics.

Keywords: eggshell quality; cuticle quality; genotype; housing system

Eggshell quality is considered a major concern in the egg industry because of the economic losses related to the incidence of eggshell defects. In egg industry, the eggshell is essential to provide the shape of the egg and as a container of the internal egg components protecting it from environmental conditions. However, these features of the eggshell are reserved by its unique structure. Mineralized eggshell is formed mainly of calcium carbonate (96%); the remaining components include organic matrix (2%), magnesium, phosphorus, and a variety of trace elements (Nys et al. 2004). From the inside outwards, the eggshell comprises of shell membranes and true shell that includes mammillary layer, palisade layer, vertical layer, and cuticle (Gautron et al. 2014). Eggshell cuticle is a very thin organic layer covering the eggshell surface and plugs the shell pores openings to limit water, gases, and bacterial penetration through the eggshell (De Reu et al. 2006). It is composed of inner

Supported by the Ministry of Agriculture of the Czech Republic (Project No. QJ1310002).

doi: 10.17221/75/2017-CJAS

calcified and outer non-calcified water insoluble layers which are deposited directly onto the vertical crystal layer of the eggshell (Kusuda et al. 2011). The structure of the eggshell is often expressed by eggshell quality characteristics including eggshell strength and thickness. These characteristics are known to be affected by several internal and external factors such as genotype of laying hens and housing systems which are considered the most important (Ketta and Tumova 2016).

Commercially available genotypes differ mainly in egg weight, shell thickness, and strength. Thus, selecting the hen genotype which provides better eggshell quality characteristics is a very important issue to be considered. The differences in egg weight according to variable hen genotypes was investigated by Zita et al. (2009) who reported a significantly higher egg weight in eggs from Hisex Brown compared to Isa Brown and Moravia BSL. Moreover, egg weight differences between Lohmann LSL and a traditional breed the Czech Hen were obtained by Tumova et al. (2016).

The eggshell strength is of utmost importance for egg producers, as the lower strength causes higher percentage of broken eggs increasing the economic losses. Zita et al. (2009) reported significantly stronger shells from Isa Brown eggs compared to Hisex Brown and the tinted-egg hybrid Moravia BSL. On the other hand, non-significant differences in shell strength were determined by Tumova et al. (2007) in variable dominant strains. The effect of hen genotype on eggshell thickness was confirmed in several studies (Singh et al. 2009; Tumova et al. 2011). Eggshell percentage might be affected by hen genotype as it differs in egg weight and eggshell weight. Tumova et al. (2016) reported that higher shell percentage was observed in Lohmann LSL eggs compared to Czech Hen. However, Basmacioglu and Ergul (2005) described a non-significant effect of genotype on shell percentage. The deposition of cuticle is influenced by a number of factors including age, genotype, egg washing, and stress. Samiullah and Roberts (2014) suggested that brown eggs have the ability to prevent bacterial penetration more than white eggs which might be related to higher cuticle deposition in brown eggs. However, studies on the effect of genotype on cuticle deposition are limited and need more investigations.

The housing system is considered as a very important factor affecting eggshell quality. Unsuitable housing systems might increase the number of broken eggs, diseases, and general stress which consequently affect the shell parameters, mainly strength.

There is a large degree of variability in the research findings on the effects of housing system on egg weight and eggshell quality parameters providing unclear indication of which production system maintains eggs with the best shell quality (Holt et al. 2011). The effect of housing system on egg weight was studied by Lichovnikova and Zeman (2008) who observed higher egg weights were produced from hens housed in cages, whereas Tumova and Ebeid (2005) reported heavier eggs were produced from litter system.

Studying the effect of housing system on eggshell strength, Tumova et al. (2011) obtained stronger eggshells in the cage housing system compared to litter. Similarly, Ledvinka et al. (2012) and Englmaierova et al. (2014) found stronger shells in cages than in litter system. Studies on the effect of housing system on cuticle deposition are very limited and need more investigations. It is hypothesized that the genotype of laying hens and the housing system might affect eggshell quality characteristics and cuticle deposition. Therefore, the aim of the present study was to evaluate the differences in eggshell quality characteristics and cuticle deposition of laying hen genotypes housed in cages and in litter system.

MATERIAL AND METHODS

Animals and conditions. Lohmann Brown, Hy-Line Silver Brown, and Isa Brown laying hens at the age of 40–56 weeks were housed in enriched cages (100 hens, 750 cm^2/hen, 10 hens/cage) and in littered pens (100 hens, 9 hens/m^2, 10 hens/pen). The environmental conditions were similar to those described by Skrivan et al. (2015). Laying hens in both housing systems were fed an identical commercial feed mixture with 15.37% crude protein, 11.58 MJ of metabolizable energy, 3.48% of calcium, and 0.56% of total phosphorous. Feed and water were supplied *ad libitum*. The daily photoperiod consisted of 14 h light, with an intensity of 10 lx at bird head level. During the experiment, eggs were collected in four-week intervals to be 660 eggs in total (20 eggs/genotype/housing system) and divided into two groups; 330 eggs were used for

doi: 10.17221/75/2017-CJAS

analyzing the eggshell quality characteristics and the other 330 eggs were used to estimate cuticle deposition.

Eggshell quality assessments. Freshly laid 330 eggs were individually weighed, length and width of each egg were measured for the egg shape index calculation (width/length × 100).

Eggshell strength was determined by the shell-breaking method using a QC-SPA analyzer (Technical Services and Supplies Ltd., UK). Eggshell thickness was measured with a QCT shell thickness micrometer (Technical Services and Supplies Ltd.) at the equatorial area after removal of shell membranes. Eggshell weight was determined after drying according to Englmaierova et al. (2015), and the eggshell percentage was calculated. The surface area of each egg was determined using the equation reported by Thompson et al. (1985):

$$\text{Egg surface area} = 4.67 \times (\text{egg weight})^{2/3}$$

Estimation of cuticle deposition. Totally 330 eggs were used for cuticle estimation by the method of Roberts et al. (2013). Eggshells were individually soaked in a MST cuticle blue stain (MST Technologies Ltd., UK) for 1 min and rinsed in tap water 3 times to remove the excess stain. The eggshell colour was measured using a hand-held spectrophotometer CM-2600d (Konica Minolta Inc., Japan) which works on the L*a*b* colour space system. L* has a maximum of 100 (white) and a minimum of 0 (black). For a*, green is towards the negative end of the scale and red towards the positive end. For b*, blue is towards the negative end and yellow towards the positive end of the scale (Roberts et al. 2013). The reading was taken 3 times per location at 3 locations around the equator of each egg and an average was recorded.

The recorded average of L*, a*, and b* values, before and after staining, was used to calculate ΔE^*_{ab}:

$$\Delta E^*_{ab} = \sqrt{[(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2]}$$

A higher ΔE^*_{ab} denotes a higher staining affinity and hence more cuticle coverage (Leleu et al. 2011).

Statistical analysis. The experiment data were evaluated with ANOVA, two-way analysis of variance using the GLM procedure of the SAS software (Statistical Analysis System, Version 9.4., 2013). The model included the effects of genotype and housing system. A value of $P < 0.05$ was considered significant for all measurements.

RESULTS AND DISCUSSION

Results of the present study showed the differences in egg weight according to the genotype of laying hens and the housing system. As shown in Table 1, Lohman Brown produced heavier ($P \leq 0.001$) eggs compared to Isa Brown and Hy-Line Silver Brown. The effect of genotype on egg weight was reported by Tumova et al. (2011) and Ledvinka et al. (2012) who detected variable egg weights from different hen genotypes. The egg weight was significantly affected by housing systems. Eggs were heavier in enriched cages ($P \leq 0.043$) compared to the litter system (Table 1). These results correspond with the findings of Englmaierova et al. (2014) who detected heavier eggs in the cage system compared to litter. On the other hand, Tumova and Ebeid (2005) and Pistekova et al. (2006) detected heavier eggs in litter systems compared to conventional cages. These conflicting results might be related to different experimental conditions and management. The eggs of Isa Brown hens were longer than those from Lohmann Brown and Hy-Line Silver Brown resulting in significantly higher egg shape index values ($P \leq 0.019$).

In literature, eggshell quality characteristics were more affected by genotype than by housing system. In the present study, eggshell strength was not significantly affected by either hen genotype or housing system (Table 1). However, differences in eggshell strength due to laying hen genotype were reported in previous studies (Zita et al. 2009; Ledvinka et al. 2012). Regarding the housing systems, no significant differences in shell strength were observed between eggs produced in litter system and cages (Pistekova et al. 2006). On the other hand, Englmaierova et al. (2014) revealed stronger eggshells produced in cages compared to litter housing system. Thus, it can be assumed that this contrast in results of eggshell strength might be related to the structure of the eggshell, especially the size and orientation of shell crystals or the mineral content of the eggshells. The relationship between eggshell strength and eggshell thickness is very important to overall shell measurements and might differ according to the thickness of the shell. Kibala et al. (2015) observed a genetic correlation between eggshell strength and its thickness was around 0.8, making the shell thickness a selection index candidate element. Ketta and Tumova (2017) indicated that

doi: 10.17221/75/2017-CJAS

Table 1. Effect of genotype and housing system on eggshell quality measurements and cuticle deposition

Genotype	Housing system	Egg weight (g)	Egg shape index (%)	Shell strength (g/cm^2)	Shell thickness (mm)	Shell percentage (%)	Shell surface (cm^2)	ΔE^*_{ab}
Lohmann Brown	cage	69.24	74.71	4027	0.358	9.48	76.67	51.24
	litter	66.60	75.85	4165	0.367	9.86	76.68	50.04
Hy-Line Silver-Brown	cage	62.55	76.31	4102	0.361	9.84	73.57	42.50
	litter	61.42	75.70	3811	0.359	10.08	72.66	40.51
Isa Brown	cage	67.41	76.61	4081	0.369	9.86	77.30	45.29
	litter	67.61	76.44	3925	0.375	10.09	77.46	42.05
RMSE		4.53	2.77	831	0.031	0.79	3.47	10.19
Genotype		0.001	0.019	0.561	0.033	0.026	0.001	0.001
Housing		0.043	0.736	0.339	0.287	0.006	0.042	0.105
Genotype * housing		0.192	0.124	0.274	0.479	0.823	0.202	0.807

ΔE^*_{ab} = calculated single score of colour difference for estimation of the degree of staining (Leleu et al. 2011), RMSE = root mean square error

the eggshell strength was significantly increased as the eggshells became thicker. Also there were different values between eggs produced in enriched cages and in litter system especially in the thin shell category, while shell strength did not differ between litter and enriched cages in the medium and thick shell categories (Ketta and Tumova 2017). In the present study, Isa Brown produced the thickest ($P \leq 0.033$) eggshells in comparison with Lohmann Brown and Hy-Line Silver Brown. Hence, the eggshell thickness was significantly affected by laying hen genotypes in spite of the non-significant effect on eggshell strength. This finding might be explained by Tatara et al. (2016) who indicated that the mechanical endurance of the eggshell is not simply affected by its thickness but by other factors, e.g. mineral density, mineral content, and spatial micro architectural arrangement contribute to this characteristic.

No significant effect of housing system on eggshell thickness was detected in the present study (Table 1). These results are in agreement with Van Den Brand et al. (2004) who found no differences in eggshell thickness in eggs from cages and outdoor system. On the other hand, Tumova et al. (2016) reported a higher eggshell thickness in cages than in litter systems between Lohmann LSL and the Czech Hen. These differences between studies might be explained by different laying hen age or the interaction of genotype and housing system. As shown in Table 1, eggshell percentage was significantly affected by hen genotype. Isa Brown eggs had the highest values ($P \leq 0.026$) compared to the other two genotypes. The effect of housing system on eggshell percentage was recorded in the present study with higher values ($P \leq 0.006$) on litter than in cages. The effect of hen genotype on eggshell surface area was noticed. The values of Isa Brown eggs were significantly higher ($P \leq 0.001$) than those from Lohmann Brown and Hy-Line Silver Brown. The results are in agreement with Anderson et al. (2004) who reported different eggshell surface area of eggs from historic strains of single comb White Leghorn.

The results of cuticle deposition indicated that the laying hen genotype plays an important role in the deposition process (Table 1). A higher cuticle coverage ($P \leq 0.001$) was in eggs produced by Lohmann Brown compared to Isa Brown and Hy-Line Silver Brown. However, housing system did not significantly affect the cuticle coverage in

doi: 10.17221/75/2017-CJAS

the present study. Samiullah and Roberts (2014) reported a significantly higher cuticle deposition in cages versus free-range eggs. This might be explained by Kusuda et al. (2011) who concluded that the diversity in the structure of the cuticle layer may be linked to the environment of the nest, mainly humidity, which is hard to control in outdoor systems. Further investigation on the effect of genotype and housing management on cuticle deposition is needed because so-far available data are limited.

Our study indicated non-significant interactions of genotype and housing system. However, several studies indicated the effect of the interactions between genotype and housing system on eggshell quality parameters to be more important than the effect of individual factors (Singh et al. 2009; Zita et al. 2009; Tumova et al. 2011).

In conclusion, the results of the present study pointed out the important effect of laying hen genotype on egg weight, eggshell measurements, and cuticle deposition compared to the lower effect of housing systems. Selecting genotypes which provide higher shell quality characteristics and higher cuticle deposition ability is very important to maintain profitability and decrease bacterial penetration and egg spoilage.

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Received: 2017–07–11
Accepted after corrections: 2017–09–21

doi: 10.17221/77/2017-CJAS

Effect of Housing System and Age of Laying Hens on Eggshell Quality, Microbial Contamination, and Penetration of Microorganisms into Eggs

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ABSTRACT

Vlčková J., Tůmová E., Ketta M., Englmaierová M., Chodová D. (2018): **Effect of housing system and age of laying hens on eggshell quality, microbial contamination, and penetration of microorganisms into eggs**. Czech J. Anim. Sci., 63, 51–60.

Hens of the laying hybrid ISA Brown were used in the study with the objective to evaluate eggshell quality, microbial contamination of eggshells, and penetration of microorganisms into the egg content in different housing systems (enriched cage: 60 hens, 10 hens per cage, 750 cm^2 per hen vs free range: 60 hens, 9 hens per m^2) and at different hen ages (26 vs 51 weeks) during storage time (0, 2, 7, 14, and 21 days). A significant interaction between the housing system and age was observed in egg weight and most of eggshell quality measurements. However, microbial contamination and penetration were affected mostly by the housing system and storage time. The numbers of *Escherichia coli* ($P < 0.001$, 4.51 vs 2.75 log cfu/eggshell) and *Enterococcus* ($P < 0.001$, 2.56 vs 1.11 log cfu/eggshell), and the total number of microorganisms ($P < 0.001$, 5.04 vs. 3.65 log cfu/eggshell) were higher in free range eggs compared to enriched cage eggs, respectively. The counts of *Escherichia coli* ($P < 0.001$, 4.23 vs 2.91 log cfu/eggshell) and *Enterococcus* ($P < 0.001$, 2.31 vs 1.27 log cfu/eggshell) decreased with storage time. A positive correlation between the total number of pores and penetration of *Escherichia coli* in both housing systems was observed in the albumen. It can be concluded that the housing system and age of laying hens significantly affected eggshell quality. Microbial contamination presumably affects the penetration of microorganisms. The correlation between the number of pores and penetration is assumed to be affected by the microbial species.

Keywords: enriched cage; free range; egg safety; hen

In the commercial egg industry, the eggshell protects the egg from mechanical damage and contamination of the internal contents. Failure of the shell for any reason compromises the value of an egg as a food product. Egg producers must be aware of these factors because the economic consequences of shell failures are significant. At the time when the eggshell is formed, all of the investment of nutrients has already been made, and the loss of nutritional value potentially represents a total loss to the farmer (Hunton 2005).

There are many factors that affect the functional quality of the eggshell, mostly prior to when the egg is laid, such as the strain, the age of the bird,

Supported by the Ministry of Agriculture of the Czech Republic (Project No. QJ1310002).

doi: 10.17221/77/2017-CJAS

nutrition, stress, disease, and the housing system. As already mentioned, the housing system has a considerable effect on eggshell quality. However, the results of the effect of the housing systems on eggshell quality are ambiguous. Eggshell quality is characterized by many indicators, such as eggshell weight, specific weight, share, thickness, deformation or strength. Major economic losses for egg producers are associated with lower eggshell strength leading to eggshell breakage. Mertens et al. (2006) reported that shell strength was the greatest in aviary eggs and the weakest in free-range eggs. Inconsistent results explainable by structural differences of the eggshell are related to the interaction of the housing system, age, genotype, oviposition time, and mineral nutrition (Ketta and Tumova 2016).

Hen age is also one of the most important factors affecting shell quality. Very young birds with immature shell glands produce shell-less eggs or eggs with a thin eggshell (Ketta and Tumova 2016). Tumova et al. (2014) detected a decreased eggshell strength in older hens in comparison with younger ones. It is likely that these structural differences in eggshell formation may also affect pore density (Tumova et al. 2011).

The safety of egg production depends on eggshell contamination and the penetration of microorganisms into the egg. De Reu et al. (2006a) reported a significant higher average eggshell contamination by aerobic bacteria and the Gram-negative bacteria of eggs from alternative housing systems compared to conventional cages. Schwarz et al. (1999) found that the number of aerobic bacteria was higher in free-range eggs than in cage eggs. Jones et al. (2002) observed that the bacterial contamination of air cells, shells, and egg contents was more common in eggs from older hens than from younger ones.

Microorganisms on the egg surface can penetrate into the egg contents. The results of a study by De Reu et al. (2006b) showed that the most frequent percentage of eggshell penetration was by *Pseudomonas* sp. and *Alcaligenes* sp. followed by *Salmonella* Enteritidis in the eggshell. These microorganisms accounted for 60, 58, and 43% of the agar-filled egg penetration, respectively. De Reu et al. (2006b) and Messens et al. (2007) proved that higher eggshell contamination led to a greater possibility of microorganism penetration and egg content contamination, which may be related with a higher contamination of eggs in alternative housing systems. Some earlier studies observed the effect of quality of eggshells on microbial penetration. Sauter and Petersen (1974) determined that bacteria of the genus *Pseudomonas* were able to more readily penetrate into whole eggs of poor shell quality. However, De Reu et al. (2006b), who compared seven selected bacterial species, concluded that the weight of eggshell or eggshell thickness had no significant effect on penetration. The effect of the number of pores on the bacterial penetration was studied by Messens et al. (2005) and confirmed that a higher penetration was detected at the blunt pole of the egg. However, De Reu et al. (2006b) did not find a correlation between the number of pores and the bacterial eggshell penetration in aerobic bacteria and Gram-negative bacteria.

Contradictory data on the effect of the housing system, eggshell quality, and penetration of microorganisms into eggs need further research. It might be expected that there is an interaction between the housing system and the other factors. Therefore, the aim of this study was to evaluate the effect of the housing system, hen age, and their possible interactions on the eggshell quality, microbial contamination, and penetration of microorganisms into eggs during 21 days of storage at room temperature.

MATERIAL AND METHODS

The experiment was approved by the Ethics Committee of the Czech University of Life Sciences Prague and the Central Commission for Animal Welfare at the Ministry of Agriculture of the Czech Republic.

The experiment was conducted with ISA Brown hens. Laying hens were housed in enriched cages (60 hens, 10 hens per cage, 750 cm^2 per hen) and in free range (60 hens, 9 hens per m^2) environments. The laying hens in the free range environment were placed in one deep-litter pen with wood shavings and with access to run. The daily photoperiod consisted of 15 h of light and 9 h of darkness. Laying hens were fed identical commercial feed mixtures N1 (with 18.7% crude protein and 11.5 MJ of metabolizable energy) from 20 to 40 weeks of age and N2 (with 15.3% crude protein and 11.4 MJ of metabolizable energy) from 41 weeks of age.

doi: 10.17221/77/2017-CJAS

Feed and water were supplied *ad libitum*. The microclimate conditions were in accordance with the laying hen's requirements (Skrivan et al. 2015).

Eggs were collected for three consecutive days in weeks 26–51 to determine egg weight and eggshell quality. A total of 150 eggs were collected from each housing system and at each age (thus totally 600 eggs were analyzed). The freshly laid eggs were individually weighed. The eggshell strength was measured using a destructive method that was performed with a QC-SPA apparatus (TSS Ltd., UK). The eggshell thickness at the equatorial plane was evaluated using a QCT micrometre (TSS Ltd.) after removing the inner and outer eggshell membranes. The eggshell weight was measured after drying at 50°C for 2 h. The eggshell index was calculated as follows: shell weight/shell surface × 100 (Ahmed et al. 2005). For the pore density determination, the shells were boiled in a 5% NaOH solution for 15 min to remove the shell membranes and then rinsed three times in distilled water. The rinsed eggshells were dried in an oven heated to 50°C. The inside surface of the shells was dyed with methylene blue. The dye solution was made by dissolving 0.5 g of 89% methylene blue crystals in 1 litre of 70% ethanol. The pores appeared as blue dots on the outside surface due to capillary action. The pore density was determined on the sharp end, blunt end, and equator of each egg. The average number of pores from three parts multiplied by the area of the egg was calculated.

The eggs for the microbial contamination analyses were also collected in weeks 26–51 of age, and 30 eggs from each housing system and each age were collected from the middle floor of the cages or from nests on the litter. The microbial analyses of the eggshell surface and the egg content were performed with fresh eggs and stored eggs at 2, 7, 14, and 21 days. The eggs were stored at room temperature (20–22°C) and a relative humidity of 55–60% on clean plastic egg cartons. A total of 120 eggs were analyzed. The numbers of *Escherichia coli* (EC), *Enterococcus* (ENT), and the total number of microorganisms (TNM) were recorded. Microbial analysis of the eggshell surface was performed according to Svobodova et al. (2015). The eggs were sampled by hand (wearing clean gloves) and placed on a clean plastic egg carton. To determine shell contamination, the eggs were placed into sterile plastic bags with 10 ml of sterile saline peptone (9 g sodium chloride, 1 g peptone, and 1000 ml distilled water) in which they were thoroughly rinsed for 2 min. A dilution series for each egg was produced by adding 1 ml of the solution (10^0, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5}). The determination of the egg content contamination was based on disinfection of the eggshell surface with ethanol and aseptic removal of the eggshell membrane and thin albumen. The microorganism analysis was conducted with standard agar methods. The number of EC was monitored using Mac-Conkey agar, the number of ENT using Slanetz Bartley agar, and TNM using Standard Plate Count agar (all Oxoid, UK). Plates with Mac-Conkey agar and Slanetz Bartley agar were then incubated for 48 h in an incubator at 37°C. The Standard Plate Count agar was incubated for 120 h in an incubator at 30°C. Typical colony forming units (cfu) on the eggshell were counted on a Petri dish after incubation. The percentages of times at which the microorganisms penetrated into the egg content were calculated afterwards.

The data were statistically evaluated using the General Linear Models (GLM) procedure of the SAS software (Statistical Analysis System, Version 9.1.3., 2003). The data for egg weight and eggshell quality characteristics were analyzed with a two-way analysis of variance (ANOVA) with the housing system and age interactions, and the data for the microbial contamination of eggshells were evaluated by a three-way interaction analysis of variance (ANOVA) with the housing system, age, and storage time interactions. All of the differences were considered significant at $P < 0.05$. The results in the tables were presented as the means and standard error of the means (SEM). The relationship between the total number of pores and penetration of the microorganisms was evaluated by estimating Pearson's correlation coefficient.

RESULTS

Egg weight and eggshell quality characteristics are provided in Table 1. The egg weight was affected by a two-way interaction ($P < 0.001$) between the housing system and age. The heaviest eggs were laid in free range at 51 weeks of age, and the lightest were detected in the same housing at 26 weeks. The egg weight was significantly higher in enriched cages compared to free range and increased with advancing age ($P < 0.001$).

doi: 10.17221/77/2017-CJAS

Regarding to eggshell quality characteristics, the two-way interaction between the housing system and age ($P < 0.001$) was observed in the eggshell strength. The strongest eggshells were found in eggs in younger hens in the enriched cage, whereas the weakest occurred in the free range and in younger hens. Eggs with significantly stronger eggshells (46.1 g/cm^2) were laid in younger hens housed in enriched cages. The eggshell thickness was only affected by the housing system ($P < 0.001$). The significant interaction between the housing system and age in the eggshell weight showed that enriched cage eggshell weight was similar in eggs from young and old hens, whereas in free range, the eggshell weight was higher at 51 weeks. Significantly heavier eggshells were observed in eggs laid in cages compared to free range and in eggs from 51-week-old hens. A higher number of pores ($P < 0.001$) occurred in free range eggs and in older hens ($P < 0.001$).

Table 2 provides the results of the microbial contamination of eggs during storage. The contamination by EC was affected by a two-way interaction between the housing system and storage time ($P < 0.001$). According to the housing system, the EC number in free range was by approximately 1.76 log cfu/eggshell higher compared to enriched cages ($P < 0.001$). The counts of EC decreased during storage ($P < 0.001$) and were approximately 1.32 log cfu/eggshell. The contamination of ENT was significantly higher in free range in comparison with the enriched cage and was not affected by age. Regarding storage time, the counts of ENT ($P < 0.001$) were the highest on the second day. The total number of microorganisms was significantly affected by the interaction between age and storage time, and the housing system higher values were in free range.

The penetration of microorganisms into the eggshell membrane and albumen are shown in Table 3. No considerable differences in the penetration of EC into the eggshell membrane between cage and free range were found. However, the penetration of ENT through the eggshell membrane differed between the housing systems. ENT penetrated the eggshell membranes in eggs from cages only in young hens during the second day of storage, whereas in free range and at the same age this occurred on the second and seventh day of storage. In older free-range hens, penetration was observed on days 14 and 21 of storage. Regarding the housing system, a higher penetration of TNM on the eggshell membrane was recorded in free range eggs compared to enriched cages; however, the influence of age and storage time was not evident. In the albumen, more frequent penetration in free range was observed for EC and TNM; however, the effect of age and storage

Table 1. Results of eggshell quality characteristics

Characteristics	Item	Age (weeks)	Egg weight (g)	Eggshell strength (g/cm^2)	Eggshell thickness (μm)	Eggshell weight (g)	Shell index (%)	Total number of pores
Housing system	enriched cage		61.0^a	46.1^a	349^a	6.17^a	8.54^a	6958^b
	free range		59.0^b	38.9^b	315^b	5.27^b	7.82^b	7454^a
Age (weeks)	26		57.5^b	43.1^a	331	5.58^b	8.12	6906^b
	51		62.5^a	41.9^b	333	5.86^a	8.24	7507^a
	enriched cage	26	60.3^b	47.5^a	347	6.15^a	8.70^a	6632
		51	61.8^a	44.6^b	352	6.19^a	8.39^{ab}	7285
	free range	26	54.8^c	38.7^c	315	5.01^c	7.54^b	7180
		51	63.2^a	39.1^c	315	5.52^b	8.10^{ab}	7728
SEM			0.214	0.340	1	0.028	0.108	39.49
***P*-value**								
Housing system			***	***	***	***	***	***
Age			***	*	ns	***	ns	***
Housing system × age			***	**	ns	***	*	ns

results of the variance analysis are indicated as significant (*$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$) or not (ns)

$^{a–c}$statistically significant differences in columns are indicated by different superscripts

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Table 2. Results of eggshell microbial contamination

Characteristics	Item	Storage time (days)	Bacterial strain log (cfu/eggshell)		
			EC	ENT	TNM
Housing system	enriched cage		2.75[b]	1.11[b]	3.65[b]
	free range		4.51[a]	2.56[a]	5.04[a]
Age (weeks)	26		3.62	2.02	4.38
	51		3.64	1.64	4.31
Storage time (days)	0		4.23[a]	2.31[ab]	4.68
	2		3.89[ab]	2.98[a]	4.53
	7		3.58[b]	1.41[b]	4.04
	14		3.55[b]	1.20[b]	4.19
	21		2.91[c]	1.27[b]	4.27
Enriched cage	26 weeks	0	3.63	1.16	3.51
		2	2.50	2.89	4.01
		7	2.96	1.26	3.89
		14	3.15	0.34	3.70
		21	1.21	0	3.16
	51 weeks	0	3.48	1.60	4.43
		2	2.96	2.01	3.60
		7	2.78	0.33	3.41
		14	2.85	1.15	3.43
		21	2.02	0.33	3.23
Free range	26 weeks	0	4.99	3.31	5.50
		2	5.06	3.61	5.66
		7	4.53	3.13	5.00
		14	4.16	2.03	4.93
		21	4.05	2.50	4.42
	51 weeks	0	4.87	3.17	5.24
		2	4.97	3.56	4.86
		7	4.04	0.87	4.03
		14	4.13	1.21	4.72
		21	4.37	2.25	5.95
SEM			0.083	0.116	0.083
***P*-value**					
Housing system			***	***	***
Age			ns	ns	ns
Storage time			***	***	ns
Housing system × age			ns	ns	ns
Housing system × storage time			**	ns	ns
Age × storage time			ns	ns	**
Housing system × age × storage time			ns	ns	ns

cfu = colony forming units, EC = *Escherichia coli*, ENT = *Enterococcus*, TNM = total number of microorganisms
results of the variance analysis are indicated as significant (*$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$) or not (ns)
[a–c]statistically significant differences in columns are indicated by different superscripts

doi: 10.17221/77/2017-CJAS

Table 3. Results of microbial penetration into the egg content

Housing system	Age (weeks)	Storage time (days)	Penetration (%)					
			eggshell membrane			albumen		
			EC	ENT	TNM	EC	ENT	TNM
Enriched cage	26	0	–	–	0.56	–	–	0.56
		2	1.11	0.56	0.56	–	–	–
		7	–	–	2.22	0.56	–	1.67
		14	0.56	–	1.67	–	–	1.67
		21	–	–	1.11	0.56	–	1.11
	51	0	–	–	1.11	–	–	0.56
		2	0.56	–	1.11	0.56	–	1.11
		7	0.56	–	1.67	–	–	–
		14	–	–	1.67	–	–	1.67
		21	1.11	–	1.11	–	–	0.56
Free range	26	0	–	–	2.22	–	–	1.67
		2	–	0.56	1.11	–	–	1.67
		7	–	0.56	2.22	0.56	–	2.22
		14	1.11	–	2.78	1.11	–	1.11
		21	0.56	–	2.22	–	–	0.56
	51	0	–	–	0.56	–	–	2.22
		2	1.11	–	3.33	0.56	0.56	1.11
		7	–	–	0.56	–	–	0.56
		14	2.22	1.67	1.11	1.67	1.67	1.11
		21	0.56	–	1.11	0.56	–	1.11

EC = *Escherichia coli*, ENT = *Enterococcus*, TNM = total number of microorganisms

time was not detected. ENT penetrated into the albumen only in free range eggs from older hens on the 2nd and 14th day of storage.

Correlations between the total number of pores and the penetration of microorganisms into the eggs are presented in Table 4. The results show a negligible relationship between the number of pores and the penetration of microorganisms through the eggshell membrane and into the albumen. Significant penetration was only observed in EC in both housing systems.

Table 4. Correlation between the total number of pores and the penetration of microorganisms

		Enriched cage	Free range
		total number of pores	
Penetration in eggshell membrane	EC	0.078	0.032
	ENT	0.178	0.017
	TNM	0.019	0.117
Penetration in albumen	EC	0.316**	0.240*
	ENT	–	0.121
	TNM	0.048	0.127

EC = *Escherichia coli*, ENT = *Enterococcus*, TNM = total number of microorganisms

Pearson's correlation coefficients are indicated as significant (*$P \leq 0.05$, **$P \leq 0.01$, *** $P \leq 0.001$)

DISCUSSION

A significant interaction between the housing system and age in egg weight was found in this study. In cages, the egg weight was increased with age within 1 g, whereas in free range, the weight increased to almost 9 g. These results are in correspondence with Van Den Brand et al. (2004), who also detected an interaction of age and the housing system, and the free range layers had eggs with lower weight than the cage layers at the beginning of the experiment; however, the egg weight in eggs from free range increased faster after 59 weeks and was greater than the egg weight

doi: 10.17221/77/2017-CJAS

in the cage. With respect to the housing system, heavier eggs were produced in enriched cage than produced from free range. Our results are in accordance with studies by Lewko and Gornowicz (2011), who also found heavier eggs in cages in comparison with free range. However, Hidalgo et al. (2008) reported that free range layers produced heavier eggs compared to other systems. In the literature, the results of egg weight in different housing systems are quite variable. These differences are probably caused by variable conditions such as genotype and feeding, among others. In agreement with Van Den Brand et al. (2004), the egg weight increased with advancing age.

All of the monitored characteristics of eggshell quality were influenced by the housing system. In the present study, stronger and thicker eggshells were laid by hens kept in cages. There was a two-way interaction between the housing system and age-affected eggshell strength and eggshell weight; however, in eggshell thickness, a significant effect was observed only in the housing system. Shell strength, one of the most important egg external quality parameters, is usually dependent on eggshell proportion and thickness. Our results were also confirmed in the study by Tumova et al. (2011) revealing stronger eggshells produced in the cage system compared with litter. Also Lichovnikova and Zeman (2008) reported higher eggshell strength in eggs from cages. The shells from eggs produced in cages seem to have ultrastructural features which support the eggshell strength. The rates of calcium deposition in shells of eggs produced in the two systems are possibly different (Tumova et al. 2011). Lichovnikova and Zeman (2008) showed that calcium content in the shell and calcium intake were higher in cages than on litter. Structural differences in the eggshell formation according to a housing system may be the result of variable pore density in eggs from cages and litter.

However, contrary to our results, Van Den Brand et al. (2004) recorded greater eggshell strength and thickness in free range eggs. Mertens et al. (2006) evaluated conventional cage, enriched cage, aviaries, and free range and found the greatest strength of the eggshell in aviary eggs, whereas the weakest was found in free range eggs. Differences in eggshell physical parameters are assumed to be related to eggshell microstructure. Differences in the eggshell structure might be indicated by the eggshell index. In the present study, the eggshell index was affected by the interaction between the housing system and age. The interaction showed differences between the housing systems at 26 weeks, whereas the measurement did not vary in older hens. Ahmed et al. (2005) noted that the eggshell index expresses the size of the crystals and the compactness of the eggshell. Smaller crystals in the eggshell are more compact and increase the strength of the eggshell. Structural differences can be associated with decreasing eggshell strength with age; however, the eggshell thickness was not influenced, which corresponds with Rodriguez-Navarro et al. (2002). The authors reported a weaker correlation between eggshell strength and thickness or weight in young hens than older ones. These changes could also be responsible for the decline of shell strength because the components of the organic matrix are involved in the control of shell mineralization and crystal orientation, and they contribute to its organization and therefore to the mechanical properties of the shell (Nys et al. 1999).

In this study a higher porosity was detected in free range eggs compared to enriched cage eggs. Similarly, Tumova et al. (2011) observed differences in the pore density between cages and litter in the equatorial area, and a higher number of pores were detected in eggs from litter. This parameter was in our study also influenced by age, with the highest values in 51-week-old hens; this was in accordance with Messens et al. (2005), who detected the highest porosity of the eggshell in the middle of the laying period. Additionally, these results may be explained by structural differences in the eggshell formation according to the housing system.

A significantly higher contamination of eggshells was found in free range compared to cage eggs in all of the monitored species of microorganisms. Our results are in accordance with a study by Belkot and Gondek (2014) who compared the microbial contamination of eggs from four different housing systems and observed a lower number of aerobic bacteria in the cage system compared to litter, free range, and the organic system. Vucemilo et al. (2010) showed that in terms of cleanliness, the cage is the most suitable system. Generally, a higher contamination of eggs by microorganisms is probably related to cleanliness (Singh et al. 2009). In alternative systems, birds move freely in their environment, and a significant amount of dust that

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originates from litter is created, which results in air contamination by microorganisms and endotoxins (Wathes 1994). In a study by De Reu et al. (2005a), the total count of aerobic bacteria in the air of poultry houses proved to be positively correlated with the initial bacterial eggshell contamination in the house. In our study, the microbial contamination of the eggshell was not affected by the age of the laying hens. However, Huneau-Salaun et al. (2010) detected that eggshell contamination increased significantly with the age of the laying hens in both flocks in cages and alternative systems. According to Mallet et al. (2003), contamination decreased with the age of hens kept in conventional and in furnished cages, but the authors attributed this decrease to a seasonal effect. However, a study by Kretzshmar-McCluskey et al. (2009) described that the microflora load on the shell increased with the age of hens.

According to the results of the present study, the microbial contamination of eggshells was also affected by storage time. The number of EC and ENT significantly decreased with time of storage, which corresponds with De Reu et al. (2005b) who observed that the total count of aerobic bacteria and the total count of Gram-negative bacteria significantly decreased within 14 days of storage time (from 4.04 to 3.23 log cfu/eggshell).

In our experiment the penetration of EC, ENT, and TNM was mainly affected by the housing system. A higher microbial penetration in the eggs from free range is assumed to be significantly affected by higher microbial contamination of the eggshells, and this assumption corresponds with Messens et al. (2007). Likewise, De Reu et al. (2007) detected a higher penetration into the egg content in eggs from an alternative housing system (2.3%) compared to eggs laid in an enriched cage (1.9%). In contrast to the housing system, the effect of age on the microbial penetration was not observed. However, Nascimento et al. (1992) reported an increasing eggshell penetration from 12.9 to 25% for *Salmonella* Enteritidis with advancing age. De Reu et al. (2006a) showed almost constant bacterial eggshell penetration during the laying period. Additionally, in this work the storage time did not significantly affect the microbial penetration. De Reu et al. (2006b) studied the influence of the storage time on the penetration of various bacterial species. Independent of the selected strain, the authors found that the eggshell penetration was observed most frequently at approximately 4–5 days. At day 6 and day 14, total eggshell penetration was up to 80% and more than 95%, respectively. The penetration of microorganisms can be affected by different factors such as eggshell quality, pore density, and others. For example, Sauter and Petersen (1974) observed that *Salmonella* more likely penetrated eggs with lower specific gravity and hence thinner shells. However, Messens et al. (2005) did not find a relationship between thickness and penetration of *Salmonella* Enteritidis. The pores of the eggshell can be the area of microbial penetration. In the present study, only a positive correlation between the number of pores and penetration was observed in EC. Board and Halls (1973) also found a correlation between the porosity and bacterial penetration. However, De Reu et al. (2006b) showed no significant relationship between the area of the eggshell, shell thickness, and the number of pores and bacterial eggshell penetration. From these contradictory results it is possible to assume that penetration may also be influenced by the species of bacteria and its activity. For instance, some types of microorganisms probably penetrate more easily than others, which was suggested by the study of De Reu et al. (2006b), in which *Pseudomonas* sp., *Alcaligenes* sp., and *Salmonella* Enteritidis penetrated most frequently compared to *Staphylococcus, Acinetobacter, Serratia,* and *Carnobacterium*.

CONCLUSION

The results of this study show the impact of the housing system and age, including their interaction, on egg weight and eggshell quality characteristics. A higher microbial contamination of the eggshell was detected in free range eggs. However, hen age had a minor effect on contamination. During the eggs storage, the number of EC and ENT gradually decreased. The penetration of bacteria into the egg content was probably related to the number of microorganisms on an eggshell surface. In addition, the positive correlation between the number of pores and penetration of EC into the albumen was observed in both housing systems. The results indicate that a relationship may exist between the quality of the eggshell and the penetration of selected species of bacteria into the egg.

doi: 10.17221/77/2017-CJAS

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Received: 2017–07–20
Accepted after corrections: 2017–11–15

6. Discussion

Studies included in this thesis work were aimed to evaluate the effect of housing system and genotype on the eggshell quality characteristics and cuticle deposition. Additionally, to study the relationship between the eggshell quality characteristics. Lastly, to estimate the eggshell microbial contamination and penetration of microorganisms into the egg content of fresh and stored eggs laid by hens of different age housed in two different housing systems.

Egg weight plays an important role as an indicator for most of the eggshell quality parameters. Ketta and Tůmová (2014) obtained significantly heavier eggs in enriched cages than those from litter. Similar observation was recorded by Vlčková et al. (2018) between enriched cages and free-range systems. Contrary, Hidalgo et al. (2008) reported heavier eggs from free-range layers compared to other systems. These conflicting results might be related to different experimental conditions and management. Moreover, the egg weight was affected by the interactions between the housing system and age (Vlčková et al., 2018). The heaviest eggs were laid in free range at 51 weeks of age, and the lightest were detected in the same housing at 26 weeks which is in agreement with published data of Lewko and Gornowicz (2011) and Englmaierová et al. (2014).

Housing system effect on eggshell thickness was not significant in the studies of Ketta and Tůmová (2018a, b). Similarly, Dong et al. (2017) and Yilmaz Dikmen et al. (2017) reported non-significant effect of housing systems on eggshell thickness. However, Ketta and Tůmová (2014) revealed thicker eggshells on litter system compared to cages in both experimented genotypes which corresponded with the findings of Tůmová et al. (2011) who indicated thicker eggshells from laying hens kept on litter system compared with those from cages, but it turned out that these shells had the lowest breaking strength. Moreover, Vlčková et al. (2018) obtained thicker eggshells in enriched cages compared to the free-range ones.

Regarding to eggshell strength, Ketta and Tůmová (2014; 2018b) indicated a non-significant effect of housing system on eggshell strength. However, in the studies of Ketta and Tůmová (2018a) and Vlčková et al. (2018) stronger eggs were produced in cages compared to litter and free-range system. Similar observations were reported by Lichovníková and Zeman (2008) and Tůmová et al. (2011) who found higher eggshell strength in eggs from cages. However, Van Den Brand et al. (2004) recorded greater eggshell strength and thickness in free-range eggs. Additionally, studying the effect of conventional cages, and free-range system on eggshell quality, Dong et al. (2017) obtained non-significant effect of housing system on eggshell strength. These conflict results might be also explained by the findings of

Ketta and Tůmová (2018a) where interactions of shell thickness category and housing system for eggshell strength were found. The eggshell strength significantly increased as the eggshells became thicker; with different values between eggs produced in enriched cages and on litter system especially in the thin shell category, while eggshell strength did not differ between litter and enriched cages in the medium and thick shell categories. The interactions between individual factors were also detected by Vlčková et al. (2018) where eggs with significantly stronger eggshells (46.1 g/cm^2) were laid by younger hens housed in enriched cages.

Eggshell thickness and strength might be related to tibia breaking strength which is an important welfare problem for laying hens. Leyendecker et al. (2001) suggested that the eggshell stability and thickness seem to be negatively correlated with the bone strength. The effect of housing system on the tibia breaking strength was found by Ketta and Tůmová (2014). The tibia strength was higher on litter system than in conventional cages which is in agreement with Lichovníková and Zeman (2008) who reported that hens kept in cages have weaker bones than those in alternative housing. Moreover, Tůmová et al. (2016) confirmed a higher tibia weight and strength in hens housed on litter compared to cages. On the other hand, Vits et al. (2005) did not detect any difference in tibia strength between the studied housing systems. Leyendecker et al. (2005) reported that, the weakness of the bones of hens kept in conventional cages is estimated to be mainly due to the limited opportunity to exercise.

The eggshell percentage was affected by housing system in the study of Ketta and Tůmová (2018a) with higher values in eggs from litter housing system compared to cages eggs. Similar results were found by Hidalgo et al. (2008) and Englmaierová et al. (2014). However, Samiullah et al. (2017) reported non-significant effect of housing system on eggshell percentage.

Laying hen genotype plays a major role affecting the overall eggshell characteristics. The results of Ketta and Tůmová (2018b) confirmed the literature published data concerning the differences in eggshell quality according to different laying hen genotypes. Eggs produced by Lohman Brown hens were significantly heavier compared to those of Isa Brown and Hy-line Silver Brown. Moreover, comparing Lohmann White and Czech Hen, Ketta and Tůmová (2014) found higher egg weights laid by Lohmann White than by Czech Hen. Similar observations were reported by Tůmová et al. (2011) who detected various egg weights from

different hen genotypes. Contrary, non-significant effect of brown, white and tinted eggs laying hens on egg weight was reported by Tůmová et al. (2017).

The eggshell strength was not affect by hen genotypes in the studies of Ketta and Tůmová (2014; 2018b). Similar results were observed by Petričević et al. (2017) (Tetra SL and Bowans Brown) and Onbaşilar et al. (2018) (Lohmann Brown Classic and Lohmann LSL Classic). However, different experiments with also brown egg hybrids (Isa Brown, Hisex Brown, and Moravia BSL) indicated significantly stronger shells in Isa and Hisex Brown eggs (Zita et al. 2009; Tůmová et al. 2011; Ledvinka et al. 2012). In spite of the non-significant effect of genotype on eggshell strength, laying hen genotype significantly affected the eggshell thickness (Ketta and Tůmová 2014; 2018b). Thicker eggshells of Lohmann White eggs than Czech Hen were obtained. Moreover, Isa Brown produced the thickest eggshells in comparison with Lohmann Brown and Hy-Line Silver Brown (Ketta and Tůmová 2018b). Non-significant effect of genotype on eggshell percentage was found by Ketta and Tůmová (2014). On the other hand, (Ketta and Tůmová 2018b) revealed that eggshell percentage and eggshell surface area were significantly affected by hen genotype, Isa Brown eggs had the highest values compared to the other two genotypes. The eggs of Isa Brown hens were also longer than those from Lohmann Brown and Hy-Line Silver Brown resulting in significantly higher egg shape index values.

Studying the effect of laying hen age on eggshell characteristics, Vlčková et al. (2018) confirmed the significant effect of laying hen age on most of the eggshell characteristics reported by Molnár et al. (2016) and Samiullah et al. (2017). Significant interactions between housing system and age for egg weight, eggshell weight, eggshell strength and shell index were observed in the study. Eggs became heavier with advancing age in free-range system compared to cages. Similar observations were reported by Van Den Brand et al. (2004) and Samiullah et al. (2017). However, eggshell strength decreased with advancing age especially in free-range system.

Regarding to egg safety, the important role of hen genotype on cuticle deposition was observed by Ketta and Tůmová (2018b). Higher cuticle coverage was obtained in eggs produced by Lohmann Brown compared to Isa Brown and Hy-Line Silver Brown. However, housing system did not affect the egg cuticle deposition. Contrary, Samiullah et al. (2014) reported significantly higher cuticle deposition in cages versus free-range eggs. This might be explained by Kusuda et al. (2011) who concluded that the diversity in the structure of the cuticle layer may be linked to the environment of the nest mainly humidity which is hard to

control in the outdoor systems. On the other hand, Vlčková et al. (2018) indicated that, the housing system play the key role regarding to eggshell contamination. A significantly higher contamination was found in free-range eggs compared to cages in all the monitored species of microorganisms. The results are in agreement with Belkot and Gondek (2014), who observed a lower number of aerobic bacteria in cages system compared to litter, free-range and the organic system. This led to a higher microbial penetration into free-range eggs. The effect of age on the microbial penetration was not detected in the study. However, Nascimento et al. (1992) reported an increasing of egg penetration from 12.9% to 25% for *Salmonella* Enteritidis with advancing age. Moreover, Huneau-Salaun et al. (2010) detected that eggshell contamination increased significantly with the age of the laying hens in both flocks in cages and in alternative systems. These results might be explained by Kulshreshtha et al. (2018) who reported a trend of lower cuticle coverage with increasing hen age.

Eggshell pores are considered as the pathway for microorganisms to penetrate into the egg content. The interactions between housing system and age were found for pores density by Vlčková et al. (2018). Higher numbers of eggshell pores were detected in free-range eggs laid by older hens compared to enriched cages eggs laid by younger hens. Similarly, Tůmová et al. (2011) reported higher pore density in eggs from litter system compared to cages. However, numerically higher pores density in cages than on litter especially in the sharp end of the eggshell were detected by Ketta and Tůmová (2014).

Vlčková et al. (2018) also revealed a significant effect of storage time on the eggshell microbial contamination. The number of *Escherichia coli* and *Enterococcus* significantly decreased with storage time, which corresponds with De Reu et al. (2006b), who observed decreasing of aerobic bacteria and the total count of Gram-negative bacteria within 14 days of storage.

The study of Ketta and Tůmová (2018a) was more focused to estimate the relationship between eggshell characteristics of eggs produced in cages and on litter using Pearson's correlations coefficients. Significantly positive correlations between eggshell thickness and egg weight in both housing systems were found. The results are in agreement with Sarica et al. (2012) who reported positive correlations between eggshell thickness and egg weight. Contrary, De Ketelaere et al. (2002) and Şekeroğlu and Altuntaş (2009) found increasing in egg weight while the eggshell thickness decreased. The results obtained by Ketta and Tůmová (2018a) also indicated a higher positive correlations between egg weight and eggshell strength. However, no correlations between eggshell strength and egg weight were found by

Şekeroğlu and Altuntaş (2009). Furthermore, Zhang et al. (2005) observed lower genetic correlations between egg weight and eggshell strength, which in turn inferred that larger eggs were not weaker than smaller ones. Positive relationship between eggshell thickness and eggshell weight were found too in the study. The results are in correspondence with the findings of Tůmová and Ledvinka (2009) and Molnár et al. (2016) who reported that the eggshell thickness was positively correlated with eggshell weight. A highly positive correlations between eggshell thickness and eggshell strength were also obtained by Ketta and Tůmová (2018a). Similarly, large genetic correlations between eggshell strength and eggshell thickness were found by Zhang et al. (2005) and Clerici et al. (2006) which explained that eggshell thickness was a major factor affecting eggshell strength. Contrary results were revealed by Tatara et al. (2016) who obtained negative correlations between eggshell thickness and eggshell strength indicating that mechanical endurance of the eggshell is not simply affected by its thickness but also by other factors such as mineral density.

The correlations between egg shape index and eggshell strength were found to be positive (Ketta and Tůmová 2018a). Similarly, Sarica et al. (2012), Blanco et al. (2014) and Gervais et al. (2016) reported that breaking strength was positively correlated with egg shape index which indicated that round eggs would show higher shell stability. However, Duman et al. (2016) found non-significant correlations between egg shape index and breaking strength. Moreover, the Pearson's correlations coefficients between eggs produced in cages and on litter showed different values especially for eggshell thickness, eggshell strength and eggshell weight (Ketta and Tůmová 2018a). A higher negative correlations between eggshell percentage and egg weight was found on litter system compared to enriched cages. While, the correlations between eggshell thickness; eggshell weight and eggshell percentage were highly positive on litter system. However, there are lack of published data concerning the correlations differences of eggshell characteristics regarding to different housing systems. Moreover, Ketta and Tůmová (2018a) reported an interactions of shell thickness category and housing system for eggshell percentage. The thin and thick shells categories showed big differences between enriched cages and litter system for eggshell percentage, while the medium shell category did not differ. These results might be related to the uniformity of eggshell thickness (Yan et al., 2014).

7. Conclusions and Recommendation for Scientific and Technical Development

Eggshell quality is influenced by a wide range of factors whereas the housing systems, genotype and age are the most important. It is highly recommended to understand how these factors influences the eggshell to maintain the quality and overall productivity.

The real choice of which housing system fit the most for laying hens lies not between an ideal, natural system and an unsatisfactory, intensive system, but between several systems, all artificial to a greater or lesser extent and all with various imperfections.

Alternative systems for laying hens should provide an environment where the hen can find and choose resources she is motivated to seek. Thus, the tibia measurements will improve and consequently improve the eggshell characteristics mainly thickness and strength. Genetic selection today provides high standard eggshells grades which are supported by more developed housing systems. For example in the thin shell thickness, housing system plays an important role in the relationship to strength. Therefore, paying attention to the interactions of factors affecting the eggshell quality should be of the outmost importance for eggs producers.

Studies pointed out the important effect of laying hen genotype on egg weight, eggshell measurements and cuticle deposition. Therefore, genotype selection provides a higher shell quality characteristics and higher cuticle deposition which maximize the egg safety. The age of the laying hen is obviously the major factor and a tremendous effort has already gone into selection to limit age-related effects on the likes of egg weight, and eggshell strength with the aim of keeping laying hens for more than 100 weeks with maintained shell quality. Achieving that goal will be of outmost benefits for egg producers.

A large number of eggs are produced worldwide for human consumption; changes in eggshell properties are directly related to increasing risk of foodborne disease for the consumers. Based on experimental and epidemiological data, it seems highly unlikely that the moving from conventional cages to non-cage systems result in an increase of microorganisms contamination and even penetration into the eggs. Therefore, it is clear that eggshell contamination with aerobic bacteria is significantly higher on average for non-cage systems as compared with furnished cages or eggs from conventional cages. In addition to the housing system, farm management also seems to play an important role in the bacterial eggshell contamination.

To summarize, regarding to the eggshell quality characteristics, the results of this thesis work indicated the important effect of individual factors represented in laying hen genotype, age

and housing system compared to the lower effect of their interactions. However, only laying hen genotype controlled the eggshell cuticle deposition. Housing system play the major role concerning the egg safety. Enriched cages are highly recommended to egg producers as it produce eggs with lower eggshell microbial contamination as well as lower microbial penetration into the egg content.

The effect of eggshell thickness on overall eggshell quality characteristics was well noticed in the studies of this thesis. Therefore, it is of outmost importance for egg producers to keep laying hens genetically selected for higher eggshell thickness. However, further future studies concerning the relationship between eggshell thickness and other shell quality parameters is required to maintain the egg industry and a safe eggs and egg products.

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