Focus on Research sponsored by the Incubation and Fertility Research Group (IFRG)

# Rooster Fertility: Diagnosis and Conservation

In poultry, far fewer males than females are involved in reproductive management programs. As a result, male selection has important consequences, particularly in terms of economic prospects and dissemination of genetic progress, depending on their reproductive capacity. Developing effective approaches for diagnosing male fertility and maintaining it over long periods of time are therefore two major challenges to improve the poultry industry.

Currently, the assessment of male fertility is based on the in vitro evaluation of semen quality, including semen volume as well as sperm concentration, morphology and motility. However, these criteria correlate poorly with male fertility observed in vivo. In our laboratory, we have developed a new strategy to diagnose sperm fertility based on proteomic methods, essentially Intact Cell MALDI-TOF Mass Spectrometry (ICM-MS), a method that can be applied directly to an isolated cell population and that is capable of rapidly characterising the intact endogenous peptides and proteins involved in various cellular functions. We demonstrated that ICM-MS applied to ejaculated sperm discriminates chickens according to their fertility status by comparing their semen protein profiles and could be used as a fertility diagnostic test based on predictive mathematical models in an experimental context.

At the same time, biotechnological methods for preserving male fertility are mostly based on sperm cryopreservation. This approach is one of the most widely used to conserve animal genetic resources, based on the collection of large quantities of cells, respect for animal welfare and the possibility of long-term storage and long-distance exchange. While several protocols have been proposed for cryoprotection of chicken spermatozoa, the use of glycerol as cryoprotectant in combination with straw packaging remains the most efficient approach for freezing chicken spermatozoa. However, the presence of glycerol in post-thawed semen samples causes a severe reduction in fertility, leading to the need to remove glycerol prior to insemination. Our recent work has described a new solution to remove glycerol from post-thawed chicken semen. This new method can be processed at room temperature, restores sperm fertility in vivo and can save 44% of the time compared to the classical removal procedure.

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## Plumage Colour Variety Effects on Body Weight and Semen Quality in Leghorn Roosters (Gallus gallus domesticus)

The aim of this study was to point out the differences between Leghorn colour varieties in order to investigate colour-based uniqueness and commonalities in a world-wide known heritage Italian chicken breed.

Semen samples collected from 50 Leghorn roosters (N=9 Silver, LHS; N=21 White LHW; N=14 Black, LHB; N=6 Gold, LHG) were analysed. Roosters (10 months old) were reared on floor following standard chicken breeder management and semen was routinely collected by the dorso-abdominal massage technique. Individual body weight (LBW) was recorded. Three quantitative (Volume VOL; Volume/LBW ratio VWR; Concentration CON) and 11 qualitative parameters (Viability VIT; Total Motility MOT; Progressive Motility PRM; Curvilinear Velocity VCL; Straight line Velocity VSL; Average Path Velocity VAP; Linearity LIN; Straightness STR; Wobble WOB; Amplitude of Lateral Head Displacement ALH, Beat Cross frequency BCF) were analysed on fresh ejaculates just after collection. ANOVA analysis was carried out using GLM procedure of SAS® 9.4 (colour variety=source of variation; Student's t-test; LSMeansSE). PCA analysis was performed using Past 4.0 statistic software. Significant differences (p 0.05) were found in LBW, VWR, LIN, WOB, ALH. In PCA analysis, LBW describes more than 99% of the variance, VCL and LIN are the qualitative parameters which better describe samples variation. LHG were the heaviest and LHW the lightest roosters (2.510.12 vs. 1.910.06 Kg), inverse proportion with LBW was recorded in VWR (LHW=0.070.01 vs. LHG=0.050.01, mL/Kg).

LIN (%) and WOB (%) were higher in LHB samples and lower in LHG samples (44.151.96 vs. 32.633.00; 65.371.55 vs. 56.612.36). On the contrary, LHG samples showed the highest values for ALH (m) and LHB the lowest: 4.050.23 vs. 3.070.15. PCA analysis reveals the differentiating effect of body weight and at the same time the effect of VCL quality parameter as second component.

Different levels of clustering ability of the varieties on the two components have been defined. Deep knowledge of breed/variety-specific features under productive, reproductive, and behavioural aspects supplies objective data for biodiversity conservation projects.

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# focus on RESEARCH

## The Effects of Light During Incubation and a Post-Hatch Enrichment on White Leghorn Layer Chick Development and Behaviour

Light exposure during artificial poultry egg incubation could be a key element underlying embryo development, post-hatch performance, and post-hatch behaviour. While previous research has primarily focused on mitigating negative effects, the influence of light during incubation in relation to positive animal welfare indicators and interaction with enrichment has received less attention. Here, we investigated the effect of light during incubation and environmental enrichment on body mass and chick behavioural responses. We quantified behavioural time budgets and activity levels, environmental usage, and interactions with the enrichment of a dark shelter. White Leghorn chicken eggs were incubated in temperature controlled photoperiodic boxes under either constant full spectrum white light (n=72; 24L:0D) or darkness (n=72; 0L:24D).

The chicks were split into 8 pens across 2 experimental rooms post-hatch, and two pens per room contained a dark shelter. Body mass and behavioural data were analysed with analysis of variance (ANOVA) using the statistical software program R version 4.2.2. Results indicate that at 4 weeks old, chicks incubated under light were heavier compared to those incubated under darkness ($p<0.05$). The dark shelter enrichment did not have an effect on chick body mass development. Light during incubation had no effect on behavioural time budgets and activity levels, but the presence of the dark shelter significantly lowered foraging ($p=0.01$), decreased eating ($p=0.01$), and increased resting ($p<0.001$) behaviour. Together these results suggest that light during incubation can affect chick growth rates and this might be a consequence of alternated metabolic and physiologic processes. Data on the dark shelter showed that light during incubation does not influence how chicks would interact with the enrichment. However, chicks use this enrichment, resulting in lower activity levels overall.

These findings have the potential to synchronise behavioural patterns and therefore mitigate behavioural problems like feather pecking. The implementation of adequate light-dark cycles into commercial practice is not a huge cost, but it could have a tremendous impact on the welfare of billions of chicks. Further research into the effects of environmental conditions in early life could improve chick health as well as enhance laying hen welfare in later life.

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### Effects of SPIDES and Preincubation Warming Profile on Embryonic Mortality and Hatchability of Long-Stored Eggs from Young Broiler Grandparent Flocks

Longer egg storage times (>7 d) are common in broiler parent and grandparent hatcheries to obtain the requested flock size. However, prolonged storage is known to decrease hatchability.

This study aimed to examine the interaction of short period incubation during egg storage (SPIDES) and preincubation warming (PW) profile after storage on embryonic mortality, and hatchability of long stored eggs. Hatching eggs were obtained from two Ross female line grandparent flocks at 29 and 30 wk of age for trials 1 and 2, respectively. In both trials, 10.800 eggs were stored for 14 d at 15°C. During the storage, the eggs were either kept continuously in the storage room (No-SPIDES) or were subjected to a SPIDES treatment, with 3.5 h above 32°C EST on d 5 of the storage period in a Petersime Re-Store machine (Figure 1). After storage, three preincubation warming profiles were used. These treatments were warming for 6 h (PW6), for 24 h (PW24) at 28°C, or warming eggs from 15°C (storage temperature) to 37.8°C in about 24 h (PWSI24). Preincubation warming procedures were conducted in a Petersime setter before incubation (Figure 2). After all treatments reached to incubation temperature, all eggs in each trial were incubated in the same setter and hatcher. In each trial, a tray of 150 eggs constituted a replicate, and 12 replicate trays (1.800 eggs) were set per subtreatment group. The data from both trials were combined and were subjected to 2-way analysis of variance (ANOVA) with trial as a block using the general linear model (GLM) procedure of SAS. Both SPIDES and long preincubation warming improved the hatchability ($P < 0.05$). However, the interaction between SPIDES and preincubation warming profile was observed for early embryonic mortality ($P = 0.047$) and hatchability of fertile eggs ($P = 0.042$). In No-SPIDES group eggs, hatchability was increased by both longer warming treatments (PW24 and PWSI24) compared with that of 6 h preincubation warming (PW6), due to lower early embryonic mortality, whereas no effect of preincubation warming profile was observed when eggs were subjected to SPIDES at 5 d of 14 d storage period. We concluded that the detrimental effects of a long storage period may be practically ameliorated by either SPIDES or by longer preincubation warming. However, the positive effect of SPIDES was more evident than the longer preincubation warming for the eggs from young flocks.

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### Chemerin Present in Egg White, Oviduct and in Embryonic Annexes During the Embryo Development in Hens: a Potential Tool for the Genetic Selection?

One of the goals of breeding companies is

# Does Vaccination Cause Stress? Comparison Between the Effects of in-ovo and Post-Hatching Vaccination on Stress Level in Chicks.

Poultry production is one of the largest and fastest-growing sectors of animal husbandry. The significant epizootic pressure increased the demand for poultry vaccinations in the hatcheries. Day-old chicks are routinely vaccinated with the manual subcutaneous injections. The in-ovo vaccination is fully automated and performed on eggs, allowing day-old chicks to be transported directly to farms immediately after sorting. The literature confirms that the in-ovo vaccination provides earlier immune protection (1) and is less stressful for the chicks (2).

The in-ovo vaccination eliminates the need for repeated vaccinations at the farm level, and thus reduces the overall stress level (3). The goal of this study is to compare the effects of the in-ovo vs. subcutaneous vaccination on the short-term stress in the embryos or day-old chicks. The experiment was conducted on the same batch of the hatching eggs for in-ovo and subcutaneous vaccination. On day 18 of the incubation the embryos were vaccinated in-ovo using Egginject (Ceva Ecat-iD). The samples were collected: blood (n=24) for glucose and corticosteroid level and pituitary gland (n=8) for the gene expression. For subcutaneous vaccination, the day-old chicks were vaccinated with Desvac Dovac (Ceva), and the sampling was repeated.

The corticosteroid is a stress hormone and biomarker for short-term stress. It was detected in the blood serum with two methods: ELISA and LC/MS. The gene expression study was based on RNA isolated from pituitary gland and it included genes responsible for activating hypothalamus-pituitary-adrenal axis (6, 7). The glucose level was significantly higher in embryos vaccinated in-ovo (196 mg/dL) vs. unvaccinated (161 mg/dL) ($P<0.05$). But, it was the same (about 188 mg/dL) in day-old chicks irrespective of the vaccination ($P>0.05$). The results of the corticosteroid levels and gene expression will be presented.

This research contributes to the knowledge on the chicken welfare, stress responses, and immunology.

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the production of viable and robust chicks. New fertility biomarkers, such as chemerin protein, may be used to improve reproductive performances in genetic program and reduce early embryonic mortality.

The aims were to (1) determine chemerin concentrations in albumen during the cycle of laying of broiler and layer hens (2) identify a potential link between chemerin concentrations in albumen and reproductive performances and (3) investigate the presence of chemerin in reproductive tract and its role in embryonic mortality. Eggs from 100 layer and broiler hens were collected during five consecutive days at three periods: before and after the laying peak, and at the end of laying. For each egg, chemerin concentration in albumen was measured by ELISA assay.

Chemerin expression in the magnum was measured by RT-qPCR. Moreover, 80 eggs were incubated to determine the chemerin expression in embryonic annexes. And, 1,200 eggs were injected with chicken chemerin antibodies (0.01, 0.1 and 1 µg/mL) at embryonic day 7 of incubation to evaluate the effect on embryonic mortality. Both breeds differed in chemerin albumen levels during their laying cycle. Chemerin amounts in albumen were positively correlated with fertility rates for layer hens ($r= 0.26$; $p= 0.01$) and negatively correlated with laying ($r= -0.51$; $p< 0.0001$), fertility ($r= -0.31$; $p= 0.03$) and hatchability ($r= -0.29$; $p= 0.01$) rates for broiler hens. Chemerin expression was higher in the magnum of layer hens compared to broiler hens. During incubation, chemerin levels in allantoic fluid were unchanged whereas significantly increased in amniotic fluid for both strains.

The inhibition of chemerin increased embryo mortality from the low dose 0.01 µg/mL antibodies for both strains ($p< 0.0001$).

The concentration of chemerin in albumen fluctuated during the cycle of laying and between breeds. This biomarker was correlated with different reproductive parameters depending of the breed. The inhibition of chemerin in egg white lead to an increase of embryo mortality suggesting a major role of chemerin in embryonic annexes for embryo growth.

The use of this new biomarker could improve reproduction rates and subsequently, contribute to economic benefits for breeding companies.

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