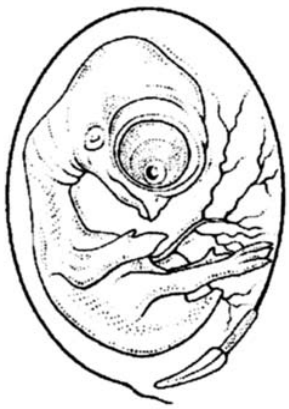

INCUBATION AND FERTILITY RESEARCH GROUP
{WPSA Working Group 6 (Reproduction)}
2012 Meeting – Pisa, Italy
13th–15th June 2012

The IFRG meeting organised by Drs Margherita Marzoni and Annelisse Castillo from the University of Pisa covered a wide range of topics from the microbiological defence systems of the egg, techniques for freezing semen, nest construction in wild birds, the long term effects of incubation temperature on chick performance and chick vocalisation during hatching. There were four invited speakers: Tommaso Pizzari (University of Oxford, UK) who discussed male behaviour and fertility; Maureen Bain (University of Glasgow, UK) describing new techniques for measuring shell quality; Jessica Dymond (USDA Beltsville, USA) who reported on experiments to improve the hatch of long stored eggs; and Ilhami Çelik (University of Selcuk, Turkey) who described the effects of incubation temperature on the development of the thymus and bursa in the chick. Dr Nick French, who has chaired the IFRG for the last 3 years, stepped down as Chairman at the meeting and Dr Marleen Boerjan (Pas Reform, The Netherlands) took over the position. It was agreed that the 2013 meeting would be held in Germany, further details would be made available on the IFRG website when they are available: www.ifrg.org

Keywords: microbiological defence systems of the egg, techniques for freezing semen, nest construction, incubation temperature, hatchability, egg shell quality, chick thymus and bursa

Physical traits of capercaillie (*Tetrao urogallus* L.) eggs and their hatchability

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In order to prevent a further decline in capercaillie (*Tetrao urogallus* L.) population, the Polish authorities decided to establish the "Capercaillie Aviary Breeding Centres" for active protection of their population. During experiments in one of such centre (in Wisla Forest District) we observed that hatchability of eggs, both hatched by capercaillie females and these incubated for 23 days by a chicken (*Gallus domesticus*) female and then moved to the incubator for the last three days, was poor. As shell quality is one of the most important egg physical traits, responsible for embryo protection against physical damage and adverse environmental conditions, O_2/CO_2 exchange and controlling water loss (Khabisi *et al.*, 2012), this study investigated the effect of egg and shell quality on capercaillie hatchability. Each egg was labelled, weighed and its shape index was determined. Post-hatched eggshells were weighed, and the shell thickness and the pore number at the blunt, equatorial and sharp end were determined.

Table 1 Characteristics of capercaillie egg morphology *versus* hatchability

Items	*n*	Value	Evaluated traits of egg morphology			
			Weight (g)	Egg dimensions (mm)		Shape index (%)
				Width	Length	
Infertile eggs	58	Mean	49.8[b]	41.3	55.0[b]	75.1
		SEM	0.44	0.11	0.27	0.37
Eggs with dead embryos	16	Mean	50.2[ab]	41.2	55.1[ab]	74.9
		SEM	1.43	0.26	0.72	0.76
Unhatched chicks	5	Mean	52.4[ab]	41.9	55.5[ab]	75.4
		SEM	1.63	0.40	0.66	0.67
Healthy chicks	63	Mean	52.2[a]	41.6	56.9[ab]	71.9
		SEM	0.51	0.12	0.32	1.30
Total	142	Mean	51.0	41.4	55.9	73.7
		SEM	0.35	0.08	0.21	0.59

[a,b] Means in columns followed by different superscripts differ significantly ($P < 0.05$).

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Table 2 Characteristics of capercaillie egg-shell *versus* hatchability

Items	*n*	Value	Evaluated traits of egg-shell				
			Weight (g)	Thickness (mm)	Pore number (pcs) on the surface of 0.25cm² in the part:		
					Blunt	Equatorial	Sharp
Infertile eggs	58	Mean	4.5[b]	0.313[a]	41.5[ab]	36.6	27.9[a]
		SEM	0.09	0.04	1.08	6.47	1.27
Eggs with dead embryos	16	Mean	3.6[c]	0.280[b]	37.6[b]	35.5	20.1[b]
		SEM	0.31	0.08	1.80	2.18	1.34
Unhatched chicks	5	Mean	7.1[a]	0.345[a]	32.6[b]	35.4	17.2[b]
		SEM	1.84	0.03	3.31	1.25	0.80
Healthy chicks	63	Mean	3.9[c]	0.275[b]	44.0[a]	37.8	22.1[ab]
		SEM	0.07	0.03	0.84	0.88	0.85
Total	142	Mean	4.2	0.294	41.8	36.7	24.0
		SEM	0.10	0.04	0.65	0.59	0.70

[a,b] Means in columns followed by different superscripts differ significantly ($P < 0.05$).

Although the infertile eggs were characterized by the lowest weight which was significantly ($P < 0.05$) lower comparing to the eggs with healthy chicks, it can be stated that the egg weight and shape index had no effect on hatchability (Table 1). The weights of other three groups of eggs did not differ significantly. The infertile eggs and eggs with unhatched chicks had the heavier eggshell than eggs of hatched and dead embryos. The same relations concern shell thickness. The pore number in the equatorial part was similar, despite egg status, however, their number in the blunt end was the highest in the eggs with healthy hatched chicks ($P < 0.05$) compared to the eggs with dead embryos and unhatched chicks (Table 2).

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Effects of *in ovo* injection of conjugated linoleic acid on hatchability and abdominal fat weight of broiler chickens

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Conjugated linoleic acid (CLA) is a collective term describing a mixture of positional and geometric isomers of linoleic acid (c18:2) involving a double bond at positions 8 and 10, 9 and 11, and 10 and 12, or 11 and 13 and may decrease the abdominal fat of chickens. An experiment was conducted to investigate the effects of *in ovo* injection of CLA on hatchability and abdominal fat weight of broiler chickens at 49 days of age. One hundred and sixty fertilised eggs including four treatments with four replicates from a young breed were selected. Fertilised eggs were injected into the air cell with CLA (150 and 300 mg dissolved in 100 mL of commercial diluent) on day 18 of incubation. Two control groups (injected with or without diluent) were also included. Results showed that although the hatchability of eggs was not affected with 150 and 300 ppm *in ovo* injected CLA ($P > 0.05$), but at 49 days of age, abdominal fat weight of chickens was linearly decreased with increasing *in ovo* injected CLA up to 300ppm dosage.

Effects of *in ovo* injection of clove essential oil on hatchability and subsequent performance of broiler chickens

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It has been reported that clove essential oil is a digestion stimulant and has strong antimicrobial, antifungal, anti-inflammatory and antioxidant activities. An experiment was conducted to investigate the effects of *in ovo*

injection of clove essential oil on hatchability and subsequent production performance of broiler chickens. A total of 200 fertilised eggs including five treatments with four replicates from a young breed were selected. The fertilised eggs were injected into the air cell with clove essential oil (50, 100 and 150 mg dissolved in 100 mL of commercial diluent) on day 18 of incubation. Two control groups (injected with or without diluent) were also included. Hatchability of fertilised eggs was recorded and subsequent mortality, weight gain, feed intake and feed conversion ratio were determined through 49 days posthatch. Results showed that the hatchability of eggs injected 150 ppm clove essential oil, was significantly higher than control groups or those injected with 50 and 100 ppm ($P < 0.05$). At the 49 days of age, weight gain of chicks was linearly increased with increasing dosage of injected clove essential oil. In conclusion, *in ovo* injection of clove essential oil up to 150 ppm dosage, had a positive effect on hatchability and subsequent growth performance of broiler chickens as compared to the control group.

Effects of maternal stress on morphological traits and mean relative asymmetry in broiler embryo and chicks

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Corticosterone (CORT) can be transferred from the mother to the egg and low levels of maternal CORT are naturally deposited into egg. Plasma CORT increases rapidly from the baseline level in response to stressor stimuli and result in a greater accumulation of CORT in the egg. Stress experienced by mothers during breeding may have consequences on offspring quality via an effect on CORT concentration in the eggs. Therefore, this study aimed to evaluate the effect of maternal stress created by CORT feeding on morphological traits and mean relative asymmetry of morphological traits in broiler embryos and chicks.

Eggs were collected from broiler breeder hens that were fed a diet supplemented with 2 mg CORT/hen/day for 14 days and a control diet. Eggs were incubated (37.8 °C and 68 % relative humidity) and embryos ($n = 13$ embryo/group) from each group were selected randomly at day 12, 14, 16, 18 of incubation and hatch. Lengths of left (L) and right (R) toe, face and shank were measured using a digital caliper. After tibia and femur bones were seperated from the muscles, bone lengths were measured from the end points. Mean lengths of toe, face, shank, femur and tibia were calculated as (L + R)/2. The mean relative asymmetry (MRA) was calculated as [(L–R)/(L + R)/2] x 100.

Although maternal stress had no effect on lengths of face, toe, tibia and femur during embryonic development, there was an interaction between maternal stress and embryonic age for face and femur lenghts on day 18. CORT embryos had shorter face and femur lengths on day 18. At hatch, tibia and femur lengths decreased in CORT chicks. The length of shank and MRA for face, toe, shank, tibia and femur lengths were higher for CORT embryos than control during embryonic development. MRA was similar in groups at hatch. Effect of maternal stress was age-specific for MRA that obtained higher during embriyonic development and similar at hatch.

Maternal stress impaired developmental stability of morphological traits during embryonic development and had an adverse effect on morphological traits, especially at 18 days of incubation and hatch. Present results suggest that maternal stress can affect morphological traits and developmental stability towards the end of embryonic development. In conclusion, stress experienced by females may negatively translate to offspring phenotypic quality.

Egg pH: where did we all go wrong?

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Just over a century ago Soren P. L. Sorensen working in the Carlsberg Laboratory in Copenhagen published a treatise in which he demonstrated the importance the hydrogen ion concentration ($[H^+]$) in the control of enzymatic processes. He expressed the hydrogen ion concentration as the negative power of 10, symbolised as pH. The introduction of the pH scale was a useful innovation as it readily described the wide range of $[H^+]$ found in biological fluids. However, it disguises the fact that the absolute concentration is actually very low, for example in blood plasma it is approximately 4×10^{-8} equiv. L^{-1}, compared with $[Na^+]$ 140 equiv. L^{-1}, $[K^+]$ 0.004 equiv. L^{-1} and $[Cl^-]$ 107 equiv. L^{-1}. In 1928, Lawrence

J Henderson formalised the relationship between pH and carbon dioxide in fluids by applying the law of mass action, so that for most of the twentieth century the acid–base status of the blood and other body fluids was characterised by the descriptive relationship embodied in what became known as the Henderson–Hasselbalch equation. But predominantly for most biologists the outcome of adopting Sorensen's pH scale was puzzlement. Then 30 years ago Peter Stewart in the US revolutionised our view of acid–base relationships (Stewart, 1981). He pointed out that contrary to the opinion of the previous 50 years the $[H^+]$ of a biological fluid was a *dependent*, not an independent, variable. If $[H^+]$ of a fluid changed then one or more of the following three independent variables must have altered: the strong ion difference (SID, $\{[Na^+] + [K^+]\} - [Cl^-]$), the partial pressure of CO_2 (P_{CO2}) or the concentration of weak acids $[A_{tot}]$. Consequently, the final pH of the fluid is not readily predictable except by a numerical solution computed from the state of six equilibria: the dissociation of water; weak acid dissociation; weak acid conservation; bicarbonate formation; carbonate formation; and electrical neutrality. The power of the Stewart analysis is that it identifies the important physiological processes that have changed pH, usually differences in SID or P_{CO2}. An example of this is found in intensive care patients where previously unidentified acid–base disturbances in need of intervention have been recognised, so improving patient survival.

The applicability of this approach to the avian embryo has been demonstrated in explanted blastoderms of the Japanese quail (Pellet-Many and Baggott, 2005). In early development ion movements and CO_2 are pivotal for the relocation of water from albumen to embryonic tissue via the sub-embryonic fluid (SEF) of the yolk sac (Latter and Baggott, 2002). Manipulation of the SID, by varying albumen $[K^+]$ and $[Cl^-]$ produced the predicted changes in SEF composition and the predicted decrease in SEF pH. Two further examples are considered here. First, the application of this analysis to the alkalosis (increased pH) of SEF of the Japanese quail observed *in ovo* around 72h incubation. Second, the physiological implications of the effect of incubating embryos in increased levels of CO_2 during the first half of the incubation period. The information that a Stewart analysis provides will be compared with the current descriptive approach.

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Developing novel tools to improve eggshell quality and egg safety

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The development of new methods for assessing a much wider range of egg properties potentially offers breeding companies new opportunities to improve the quality and safety of their eggs whether they are being consumed or set for hatching. In this paper, two novel measurements of assessing egg quality are described along with their potential application in selection programs.

The cuticle is the proteinaceous layer which covers the surface of the hen's eggshell. It has been suggested that this layer forms the eggs first line of defence to the trans-shell penetration of micro-organisms which maybe present in the cloaca or in the external environment after oviposition. A reliable method for the quantification of cuticle deposition has, however, only recently been devised. This involves measuring the difference in percent reflectance with a spectrophotometer at 650 nm before and after staining eggs with a 1% (w/v) aqueous solution of Edicol Supra Pea Green H dye. In pedigree laying flocks, cuticle deposition has been shown to have a moderate heritability (0.27) which is well within the range for traits that are currently used for selection. The natural variation in this measurement has also been shown to have a significant effect on whether *E. coli* enters the egg contents. This suggests that the vertical and horizontal transmission of zoonotic and pathogenic organisms could be reduced, and the health of poultry and the safety of table eggs improved, by selection for cuticle deposition.

A rapid and efficient method based on the analysis of two dimensional X-ray diffraction (2D-XRD) patterns for measuring the average crystal size and orientation of the calcium carbonate ($CaCO_3$) crystals in eggshells is also now available. These microstructural properties are believed to significantly contribute to the mechanical strength of the eggshell. The heritability estimates for the 2D-XRD measurement of $CaCO_3$ crystal size has been shown to be high (0.6) whilst that for crystal orientation moderate (0.3) in layers. A strong genetic correlation between these microstructural properties and shell thickness, and

in particular with the thickness of the mammillary layer, has also been demonstrated. This is consistent with the hypothesis that the structural organisation of shell, and in particular the mammillary layer, is influenced by crystal size and orientation especially during the initial phase of calcification. Correlations with shell breaking strength however were positive which is contrary to expectation.

The formation of gas exchange pores and hence water vapour conductance across the shell is determined by the number and arrangement of mammillae per unit area and the effect this has on the dimensions of the crystal columns which make up the palisade layer. The mammillary layer is also from where the developing chick derives the majority of its calcium for skeletal development after the 12th day of incubation. The microstructrual properties of the eggshell possibly influenced by matrix proteins may therefore play a critical role in the successful development of the chick embryo. The magnitude of the genetic component for crystal size and orientation could therefore present a potential route to improving embryo fitness.

Microbial environment of the hen modulates the innate immune defence of the egg against bacteria

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The egg is a complex formation that allows the development of an embryo *ex utero* by providing essential nutrients and a molecular protective system. In addition to immunoglobulins, molecular defences of the egg are also composed of antimicrobial peptides/proteins that are predominantly localised in egg white (EW). Recently, numerous antimicrobial proteins have been identified in the various egg compartments (Réhault *et al.*, 2011). These peptides and proteins are secreted as a preventive protection by the hens. They are present at low concentrations and might be modulated either by genetic (Sellier *et al.*, 2007), environment of the hens or storage conditions of the egg (Réhault *et al.*, 2010). The present study investigated whether the innate antimicrobial protection is stimulated by the degree of contamination of the hen milieu by using three experimental models. Firstly, we compared the global antimicrobial activity of EW issued from hens reared in extreme bacterial environments: bacterial free (axenic, Ax), specific pathogen free (SPF) and standard (ST) conditions. EW issued from the ST and SPF groups demonstrated a greater inhibitory effect against *Staphylococcus aureus* as compared to those of Ax groups (9.8% and 4.0% respectively, $P<0.05$). The second approach explored the effect of an immune challenge in hens on the antimicrobial potential of EW. EW from hens stimulated with *Salmonella* Enteritidis lipopolysaccharide showed greater antimicrobial activity against *Staphylococcus aureus* (14.8%, $P<0.001$) than those of control hens. Finally, we tested the effect of vaccine against virus (BI), or parasite (coccidiosis) and showed a stimulation of eggwhite antimicrobial activity in hens following the virus vaccine. These results suggest that hens can reinforce the antimicrobial activity of the EW in response to immune stimulations induced by microbial contamination and anticipate the needs of the embryo in terms of protection. Further quantitative analyses of magnum gene expression and EW concentration of antimicrobial protein candidates were carried out to explore putative mechanisms of regulation.

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Intraspecific variation in ostrich sperm velocity and viability in response to seminal plasma

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Seminal plasma may play a significant role in sperm survival and, therefore, should be carefully considered in the development of a semen extender for assisted reproductive technology. We examined the viability and velocity of sperm of seven ostriches after reconstituting centrifuged semen with: (1) seminal plasma of the same male (SPS); (2) seminal plasma of another male (SPD), and (3) Dulbecco's Modified Eagles Medium (DMEM). Semen was collected daily (09.00 to 19.00) from one or two pairs of males, using the dummy female method, and each pair was replicated once. After incubation for 15 min at room temperature, sperm motility was video recorded and the average path velocity (VAP) of each sample was estimated using a computer assisted sperm analysis (CASA). The nigrosin–eosin staining protocol was used to determine the proportions of live normal, live abnormal and dead sperm. The volume of neat semen (mean ± SE) was 1.24 ± 0.11 mL, and the live normal, live abnormal and dead sperm in the neat semen were 71.2 ± 2.44 %, 16.74 ± 1.32 % and 12.24 ± 1.62 % respectively. Re-suspending spermatozoa in DMEM resulted in a lower number of live normal (30.63 ± 2.49%), live abnormal (12.37 ± 0.71%) and a higher number of dead sperm (57.88 ± 2.85%) than in SPS or SPD ($F_{2,112} = 10.79$, $P = 0.001$). No inter-male variation between media was observed ($p > 0.05$). However, while re-suspending sperm in these three different media did not affect their velocity (VAP median: $F_{2,112} = 1.02$, $P = 0.366$), inter-male variations were observed in terms of the response of their sperm to different media ($F_{14,112} = 2.62$, $P = 0.002$). While sperm from some males had higher velocity in SPS, others showed lower velocity in SPS, as compared to DMEM or SPD. These results suggest that sperm benefits from seminal plasma but because male ostriches differ in the composition of their seminal plasma sperm of some males may not be compatible with seminal plasma from other males. Therefore pooling of ejaculates for either short storage or freezing may compromise sperm viability. Moreover, semen of particular males might be adversely affected during short liquid storage or freezing process when re-suspended in the seminal plasma of other males. Further studies are required to investigate the composition of seminal plasma to provide a better understanding of how seminal plasma influences sperm in this species.

Changes in the air cell volume of artificially incubated ostrich eggs

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Egg candling is commonly used on commercial ostrich farms during artificial incubation to determine fertility and to monitor progress of the developing embryo, but in most cases the latter is not very effective. In this study, we report on aspects of water loss of artificially incubated ostrich eggs, by assessing changes in air cell volume and factors influencing it during the incubation period. A total of 2 160 images of candled, incubated ostrich eggs were digitized using the software package AnalySIS® (Soft Imaging System, 1999), to determine the percentage of the egg volume occupied

by the air cell at different stages of incubation. The volume occupied by the air cell (measured in pixels as determined with AnalySIS® was expressed as a percentage of the volume occupied by the entire egg (also expressed in pixels; Figure 1).

Examples of images acquired in this way for embryos of 3 to 42 days of age, are depicted in Figure 1. The air cell occupied 2.46% of the volume of fresh eggs. For eggs that hatched successfully, this increased to an average of 9.31% at 15 days of incubation, 11.2% at 22 days of incubation, 13.8% at 29 days of incubation, 15.18% at 36 days of incubation and 24.4% at 41 days of incubation, just prior to hatching. Air cell volume from 29 days of incubation for infertile eggs (19.3%) was significantly ($P < 0.05$) higher when compared to dead-in-shell eggs (14.3%) or eggs that hatched (13.8%) (Figure 2). No clear differences among the different categories of eggs (hatched, dead-in-shell or infertile) were found at hatching.

Air cell volumes were largely independent of strain (South African Blacks or Zimbabwean Blues) or whether chicks were assisted to hatch or not. At 41 days of incubation there was a significantly greater ($P < 0.05$) air cell volume in eggs that hatched normally compared to dead-in-shell eggs (28.3% *versus* 21.7%, respectively). No significant differences in air cell volume were observed up to day 20 of incubation between eggs that exhibited high, average or low rates of water loss. However, for the dead-in-shell eggs, air cell volume was consistently higher during mid-incubation in eggs that exhibited high rates of water loss when compared to hatched eggs (Figure 3).

Although some subtle differences were detected between hatched and DIS chicks during this study, these differences were too small for the techniques to find application in the broader industry.

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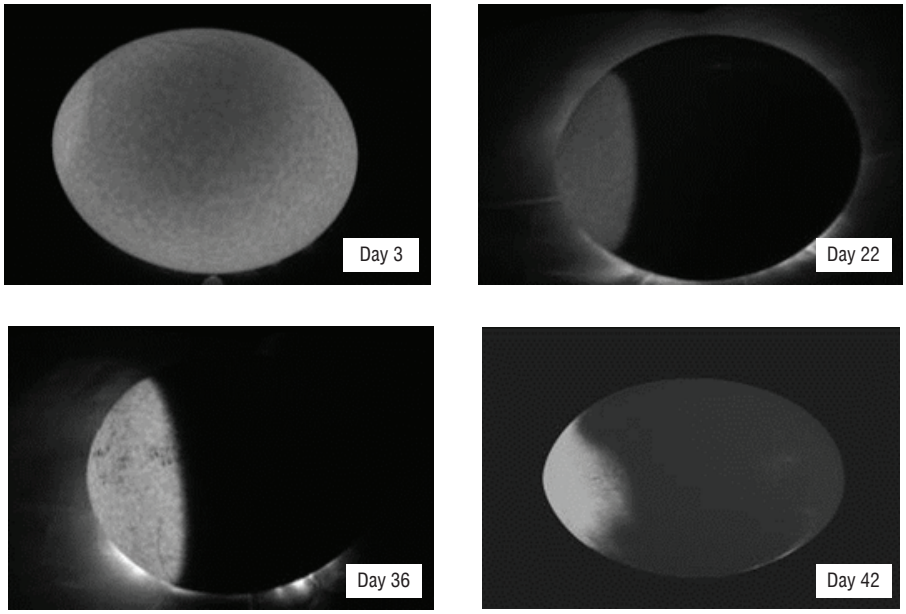

Figure 1 Images of the ostrich egg used to derive the percentage of the candled eggs occupied by the air cell from 3 days to 42 days of incubation.

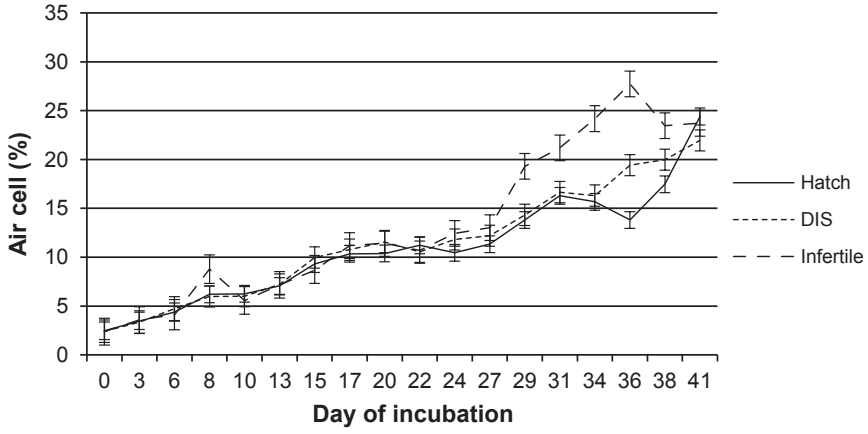

Figure 2 The percentage of the egg volume (± S.E.) occupied by the air cell in eggs that hatched (Hatch), infertile (Infertile) and dead-in-shell (DIS) ostrich eggs during the 41 day incubation period.

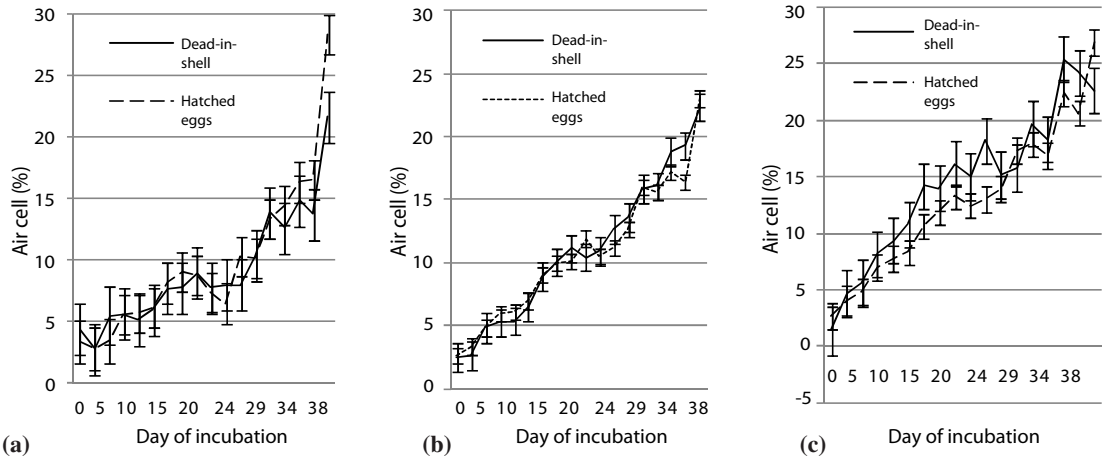

Figure 3 The percentage of the egg volume occupied by the air cell in dead-in-shell and hatched ostrich eggs during the 41 day incubation period for eggs with low (a), medium (b) and high (c) levels of water loss.

Dietary manipulation and sensitivity to cryopreservation in chicken spermatozoa

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Semen production and quality can be affected by dietary manipulation in the chicken (Cerolini *et al*., 2006). The aim of the experiment was to study the sensitivity to cryopreservation of chicken spermatozoa enriched with vitamin E and n–3 long chain polyunsaturated fatty acids by dietary manipulation.

ROSS broiler breeders ($n = 18$) were housed in single cages in controlled environment and fed the following treatment diets: C) control with 100 mg vitamin E kg^{-1}; CE) control diet + 200 mg vitamin E/kg; CEF) CE diet + 1% fish oil. Semen samples were routinely collected by abdominal massage twice per week. Semen volume and concentration were recorded in all ejaculates. Then, ejaculates were diluted to 1.5×10^9 sperm mL^{-1} in pre-freezing diluent and processed for freezing according to the pellet procedure previously described (Cerolini *et al*., 2008). Semen quality was assessed before and after freezing/thawing. The proportion of sperm with damaged plasma membrane was assessed by the modified fluorometric ethidium bromide exclusion procedure using hypotonic solution (stress test). The proportion of motile sperm and motility parameters (VCL, STR, LIN, ALH, VSL, VAP) were assessed by computer assisted semen analysis (Hobson sperm tracker system, UK). Analysis of variance was performed using GLM procedure of SAS and dietary treatment was used as source of variation. The recovery rate of undamaged and motile sperm after cryopreservation was calculated.

Dietary treatments significantly affected sperm production (semen volume and concentration) and a general significant increase in total sperm output per ejaculate was found in group CEF (1.11×10^9) compared to group C (0.84×10^9) and CE (0.68×10^9). The proportions of sperm with damaged plasma membrane and of motile sperm in fresh ejaculates were not affected by dietary treatments and the mean values recorded were 6.4% and 63.1% respectively. Dietary treatments significantly affected sperm motility parameters: mean values for STR (%), LIN (%) and VSL ($\mu m\ s^{-1}$) were significantly higher in CE group compared to C group. In contrast, sperm motility parameters recorded in C and CEF group did not show significant differences. Semen quality was greatly reduced after freezing/thawing and no significant differences were found between dietary treatments in thawed semen samples. The mean recovery rate of sperm with undamaged plasma membrane and of motile sperm after freezing/thawing was 21% and 46% respectively. No significant differences were found between dietary treatments for any of the sperm motility parameters recorded in thawed semen.

In conclusion, dietary supplementation with vitamin E was effective in increasing the quality of sperm motion in fresh ejaculates; however, such a positive effect was not still present after freezing/thawing procedure. Dietary supplementation with both vitamin E and fish oil increased sperm production but did not affect semen quality in fresh and frozen/thawed ejaculates.

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Egg production, fertility and hatchability in the Italian chicken breed *Milanino*

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Local chicken breeds are a vital reservoir of gene resources and their conservation has a technical role related to the future development of the productive system, as well as a social-cultural role. In Italy, 90 local avian breeds were described, the majority (61%) were classified as extinct and only 8.9% still used on local farms. Therefore, efforts for conservation of Italian avian breeds are urgently required. The aim of this study was to record the breeding performance of the Italian local breed *Milanino* and multiply a small population in order to develop a conservation program.

Milanino is a chicken composite breed selected at the beginning of the 20th century in Lombardia and was largely used on local farms mainly for meat production. *Milanino* breed is currently included in a conservation project run by the University of Milan (CoVAL project funded by Regione Lombardia in 2011). The breed has beautiful plumage perfectly white and soft, white skin and simple comb. The rooster has beautiful shape with a massive chest size; the hen has extraordinary fecundity and reduced space requirements. The mean body weight is 3.5 kg and 2.5–3 kg for adult males and females respectively.

Seventeen females and four males were kept at the Poultry Unit (Faculty of Veterinary Medicine, Lodi) during the reproductive season (January-June) in 2011. Birds were divided into four families (one male/four or five females) housed in floor pens in controlled environment. Birds received a photoperiod of 15L:10D and were fed *ad libitum* a commercial standard breeder diet. Egg production was recorded daily. Eggs were weighted and stored until settings every two weeks. Eggs were classified in three egg storage groups: 1 = 0–5 days, 2 = 6–10 days and 3 = 11–15 days. Standard incubation parameters for chicken eggs were used. Fertility and early embryo mortality were recorded on the 7th day of incubation by candling. Hatchability and late embryo mortality were recorded.

Mean egg production recorded from January to June was 58% and the peak of oviposition, corresponding to 82%, was recorded in mid-March. Mean egg weight was 59 ± 4 g. Six consecutive incubations were performed from February to June. High fertility values, from 85 to 91%, were recorded in all incubations. The highest hatchability values were recorded in incubation 1 and 2, 69 and 66% respectively, and a progressive decrease was found in the subsequent incubations. The length of egg storage significantly affected hatchability. The mean hatchability value recorded in eggs stored up to 5 days was 72%, and it was greatly progressively decreased in eggs stored from 6 to 15 days.

The present results contribute to the knowledge on reproductive parameters necessary to improve the reproductive efficiency of *Milanino* breed within the conservation project.

Effect of latitude on nest construction and function in Great Tits (*Parus major*) and Blue Tits (*Cyanistes caeruleus*) in Great Britain

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The function of bird nests seems to be quite straightforward – they contain eggs and/or chicks during incubation and/or rearing. Their construction could be considered in simple structural terms in the role of keeping eggs/chicks safe and secure. What is apparent is the fact that nests are constructed at a time when the bird has no eggs or chicks. Does the bird construct a nest that anticipates the future requirements of its clutch or are other factors involved? It has shown in Great Tits (*Parus major*) and Blue Tits (*Cyanistes caeruleus*) that the proportion of nest components change between breeding seasons and that in the latter species the temperature immediately before first egg date, *i.e.* the time when the clutch is initiated and the nest construction is being completed, exhibits a significant negative correlation with nest mass (Britt and Deeming, 2011). Moreover, in Canada nest construction

is affected by latitude with birds in colder northern Manitoba constructing more substantial nests than conspecifics nesting in south-eastern Ontario (Crossman *et al*., 2011). This strongly implies that nest construction is actually a function of the parent bird's thermoregulatory requirements rather than those of the eggs or chicks it produces. If it is cold when a nest is being constructed then a more substantial, presumably warmer, nest is built compared to times when the ambient temperature is warmer.

This study (see Mainwaring *et al*., 2012) examined this hypothesis by collecting nests of Blue tits and Great tits from nestboxes at seven different locations in Great Britain from Penryn in the south to Edinburgh in the north. This represented 5° of latitude and 580 km in distance. Dry nests were weighed intact and then their insulatory properties were measuring by recording the difference in cooling of *iButtons* placed in either ambient air or in the cup lining material. Thereafter, the nest was partially deconstructed by removal of the nest lining material and this and the main structural component were weighed. Mean spring temperature data were data were obtained from the UK Meteorological Office.

More northerly latitudes were cooler than southerly latitudes. For both species there were significant negative correlations between mean temperature and nest cup mass but no relationships with nest structural components (mainly moss). The insulatory properties of the nest cup also showed a negative relationship with temperature for both species. If temperature was restricted to the seven days immediately prior to initiation of the clutch then these patterns were replicated except for insulatory properties of Great tit nests, which were no-significant.

These data support the hypothesis that nest construction reflects the prevailing temperature at the time and so is more attune with the thermal properties of the incubating bird. Further study will examine these nests to determine whether they vary in their components and the study is being repeated for the Common Blackbird (*Turdus merula*), an open nesting species that is presumably highly sensitive to the prevailing temperature.

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The effect of short periods of incubation during long egg storage on the development of the broiler embryo

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Standard commercial practices necessitate the cold storage of fertilised chicken eggs prior to incubation to accommodate competing demands on space and labour, as well as to coordinate large hatches. While brief storage at 16°C does not have a detrimental effect on hatch rate, prolonged storage over ten days decreases both hatch rate and chick quality. The SPIDES (Short Periods of Incubations During Egg Storage) treatment, 4h 37.5 °C incubations delivered at four time-points over prolonged 16°C storage, increases the hatch rate of stored fertilised eggs; however, the biological basis for this phenomenon is not well-understood. To examine developmental changes underlying this effect, we analysed hatch rate, developmental stage progression, and cell viability in treated (SPIDES), untreated (non-SPIDES, stored 22 days prior to incubation), and control embryos (stored four days prior to incubation) from a commercial broiler line. The control hatch rate, averaging 89.8%, was reduced by prolonged storage to an average of 71.9%; SPIDES treatment restored the stored hatch rate to an average of 76.6%. SPIDES embryos advanced from an approximate developmental stage of Eyal-Giladi-Kochav stage X (oviposition) to Hamburger-Hamilton stage 3 (primitive streak formation). Cell viability did not differ dramatically between SPIDES and non-treated (non-SPIDES) embryos, suggesting the primary benefit of the SPIDES treatment is developmental stage progression and not maintenance of live cells in the stored embryo. The SPIDES regimen is an effective intervention in long-stored embryos exhibiting a catastrophic decrease in hatchability (less than 50% hatch rate); it will be interesting to determine if the role of SPIDES in promoting survival of these embryos also depends upon developmental stage progression or if maintenance of a population of viable cells is more important under these conditions. Current efforts are underway to describe the effect of SPIDES on stress and programmed cell death in the long-stored chicken embryo.

Some biochemical changes in blood and tissues of one-day-old chicks exposed to cadmium during embryogenesis

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Cadmium (Cd) is known to cause teratogenesis in a wide variety of animals and has adverse effects on fertility and early embryo development of birds, so avian eggs and embryos are considered to be sensitive indicators of heavy metal contamination. The aim of the study was to investigate the *in ovo* exposure effects of cadmium on developing hen embryos by monitoring some biochemical parameters of newly hatched chicks.

The fertile hen eggs were injected with Cd and the mortality during incubation were measured. Briefly, 50 μL of saline solution, containing Cd ions at a concentration from 0 (control group) to 24 μg, was injected into egg albumen on day 4 of incubation. Immediately after hatching, chicks were weighed and blood and tissues were collected. Blood plasma was assayed spectrophotometrically for the activity of lysosomal enzymes (seven glycosidases and arylsulfatase). The plasma mineral profile was determined by semi–automated clinical chemistry analyser Microlab 300. The activity of N-acetyl-β-D-glucosaminidase (NAG), chosen as biomarker of Cd toxicity, was determined in tissue homogenates. The cadmium distribution in tissues was examined by LA-ICP method.

The results showed that giving cadmium at doses exceeding 1 μg/egg caused a gradual decrease in hatchability, with LD_{50} of 3.9 μg/egg (Dżugan *et al*., 2011). Moreover during exposure to increasing Cd dose more males than females were hatched. Compared to the control group, in the blood plasma of chicks from the groups receiving 3, 6 and 12 μg Cd/egg an increase of some lysosomal enzymes activity were observed; the most significant ($P<0.05$) for NAG activity which increased by 79, 108 and 54% *versus* control group (Dżugan *et al*., 2011). Generally, cadmium did not affect the mineral composition of blood plasma although significant ($P<0.05$) changes in plasma magnesium concentration were observed.

Cadmium-related changes in the activity of NAG in tissues of hatched chicks were observed irrespective of sex; for the dose 6μg per egg the activity was increased by 25 and 35% in liver and kidney, respectively. However Cd-induced changes in NAG activity were more intensive in ovaries than in testes. The LA-ICP examination confirmed that the cadmium accumulation occurred in liver and kidney, furthermore enhanced Cd level in testis and ovary tissues was observed.

The results confirmed the high toxicity of cadmium for avian embryos. It was demonstrated, using NAG as a biomarker that that destabilisation of lysosomal membrane may be one of the mechanisms of cadmium cytotoxicity.

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Effects of silver nanoparticles administration on reproductive performance of Japanese Quail

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Silver nanoparticles are being used in Iran as a disinfectant in poultry production farms. Although many studies have been done to evaluate the impact of nanosilver in birds, the effects exerted by silver nanoparticles administration on the overall reproductive performances of avian species has not been fully studied. The present study investigated the effects of silver nanoparticles administration on reproductive performance of Japanese Quail. A total of 24 males ($n=1$ per replicate) and 72 females ($n=3$ per replicate) were randomly assigned to one of four treatment groups, six replicates per group. Three females were housed with one male in a cage pen.

The birds were supplied with commercial laying quail diets and tap drinking water *ad libitum*. Four treatments of 0, 4, 8 and 12 ppm of nanosilver were used in the drinking water from one day to 30 weeks of age. Egg production and egg quality characteristics were studied. Although the administration of silver nanoparticles decreased hen-day egg production, it significantly increased fertility and hatchability of eggs ($P < 0.05$). Egg weight was not significantly affected by administration of nanosilver, but there was a positive relationship between levels of silver nanoparticles and yolk:albumen ratio ($P < 0.05$). There was also a correlation between levels of silver nanoparticles and the **weight of the day-old Quail chicks** ($P < 0.05$). These results suggest that using supplemental silver nanoparticles during the production period can affect the reproductive performance of Japanese Quails. It can improve yolk:albumen ratio, the **weight of the day-old chicks**, **hatchability and fertility of** Quail eggs but administration of silver nanoparticles decreased egg production.

Incubation research: What do we know and what do we need to know?

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In 1990, Tullett suggested that the basic principles of artificial incubation were well understood and that poultry hatcheries were generally getting good results; future studies would just be a fine-tuning the process. The major change since 1990 has been the understanding that the requirement for incubation needs to be defined at the level of the egg rather than the incubator. The environment for an incubator needs to ensure that the eggshell temperature is maintained at 37.8ºC and the eggs lose 11 – 12% of their fresh egg weight due to water loss by the day of transfer. More recent studies have also shown that carbon dioxide levels should increase up to mid-point of the incubation period and thereafter decrease, although further studies are needed to confirm this.

A second development in incubation research since 1990 is the understanding that the incubation environment does not only impact on hatching success but also the posthatch performance of the broiler chick. In the last 5 years, there has been a significant rise in the number of papers published on incubation and to a large extent this research has looked at the effect of incubation on posthatch performance. Sub-optimal incubation temperature has been shown to depress subsequent broiler growth and resulted in changes to the development of the heart, skeletal, gut and immunological systems. The economic consequences to the poultry industry of poor broiler performance are many fold greater than the effects of hatch performance and as a consequence of the new research the industry is placing a greater emphasis on optimising the incubation environment.

In addition to showing that sub-optimal incubation can adversely impact broiler performance, other research is suggesting that targeted short-term changes to the incubation environment can have a permanent and positive effect on the development. Short periods of high incubation can improve the abilities of broilers to withstand heat stress later in life, while cool temperatures can improve resistance to ascites. In turkeys, high and low temperatures mid incubation have been shown to alter the number fibres in the muscle suggesting a possible long term effect on meat quality. The possibility that the incubation process can be used to modify the broiler in a positive way so that it is better adapted to its environment or produces a better product for the consumer is a truly exciting development and will be a growing area for future research.

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A study on the cryopreservation of Muscovy drake semen

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Despite extensive research on poultry semen cryopreservation, there is limited success in its commercial application. The sperm cryopreservation for *ex situ* management of genetic resources in poultry is still a big problem and cryobanking for species other than the chicken remains extremely limited (Blesbois and Brillard, 2007). The cryopreservation of Muscovy drake semen is more difficult than Pekin drakes (Tselutin *et al.*, 1995).

The choice of diluents and cryoprotectants and their use and effectiveness are one of the most important factors for success of the cryopreservation process. The aim of this experiment is to examine the efficacy of AU extender and five cryoprotective agents on the cryopreservation of Muscovy drake spermatozoa.

Semen was collected from individual males by placing the female with an artificial vagina in the cage of the Muscovy drake, two times per week. The pooled semen was diluted at 1:3 ratio (semen:diluent) with AU extender and added to egg yolk in a concentration of 15 % (v/v). The semen was equilibrated in a refrigerator at 4 ^{0}C for 30 min without cryoprotectants. The diluted semen was distributed equally in 5 sterile glass tubes again containing either glycerol, ethylene glycol, diethylene glycol, polyethylene glycol or thiodiethylene glycol in 5 % as a final concentration respectively. The semen samples were equilibrated in a refrigerator at 4^{o}C for 30 min again, and then they were directly dropped in concave cavities of dry ice at –79^{o}C for 10 min. The semen pellets were placed in an atmosphere of liquid nitrogen for 5 - 10 min and finally they were put in cryotubes and plunged into liquid nitrogen (LN_2). The pellets were kept frozen in the LN_2 container for two months. The semen samples were thawed with AU extender (1:3 v/v) at 42^0 C. The sperm mobility was assessed using a Nikon Alphaphot-2YS2 microscope (10×40) and abnormal and dead spermatozoa (%) by supravital eosin/nigrosin staining. The cryopreservation semen was stored once again for 6 years in LN_2 container and it was evaluated by Sperm Class Analyser Micropticum (Spain).

The results showed that the semen parameters of the equilibrated semen with 5% cryoprotectant changed significantly ($P<0.05$) compared to those in the fresh semen and in the equilibrated semen without cryoprotectant (Table 1). The unfrozen semen with 5% glycerol had the best sperm mobility and the lowest levels of abnormal and dead spermatozoa *versus* the semen with other cryoprotectants. This our result confirms our previously experiment with using other cryoprotective agents and semen freezing into plastic straws, that glycerol is a good cryoprotectant. Two-months-stored and six-years-stored semen samples with glycerol showed similar levels of sperm mobility and percent of abnormal and dead spermatozoa. Computer-assisted sperm motion analysis (CASA system) indicate that in the equilibrated semen without cryoprotectant VCL was 104.61 ± 10.31μm s^{-1}; VSL, 27.97 ± 2.93 μm s^{-1}; VAP, 54.52 ± 5.26 μm s^{-1}; LIN, 26.72 ± 0.47 %; STR, 51.25 ± 0.95 % and WOB, 52.14 ± 0.10 %, while in the unfrozen semen VCL was 39.55 ± 4.50 μm s^{-1}; VSL, 17.99 ± 1.65 μm s^{-1}; VAP, 27.87 ± 2.65 μm s^{-1}; LIN, 45.78 ± 2.86 %; STR, 64.59 ± 0.80 % and WOB, 70.82 ± 3.60 %.

It seems that Muscovy drake spermatozoa are more sensitive to the freezing-thawing process. Glycerol is a good cryoprotectant compared wih the alternative options.

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Table 1 Characteristic of fresh, equilibrated and frozen-thawed semen

Semen samples	Sperm mobility(%)	Viable abnormal spermatozoa(%)	Dead spermatozoa(%)
	$X \pm S_X$	$X \pm S_X$	$X \pm S_X$
Fresh undiluted semen	78.33±1.91	9.33±1.49	7.67±1.05
Equilibrated diluted semen without cryoprotectant for 30 min	74.50±1.46	11.17±1.25	10.33±0.88
Equilibrated diluted semen for 30 min with 5 % content of:			
• glycerol	61.67±1.83	17.00±0.85	16.83±1.04
• polyethylene glycol	57.00±2.28	18.50±1.23	18.17±1.11
• thiodiethylene glycol	54.17±3.29	21.50±1.87	20.68±0.61
• ethylene glycol	57.00±3.28	18.83±1.75	18.67±1.43
• diethylene glycol	27.50±3.39	25.50±2.15	33.17±2.61
Thawed semen with 5 % content of:			
• glycerol	41.40±4.93	25.40±1.04	40.80±2.41
• polyethylene glycol	9.00±2.55	47.00±5.79	47.33±2.16
• thiodiethylene glycol	0	0	100
• ethylene glycol	8.25±4.12	21.75±9.33	70.00±12.76
• diethylene glycol	0	0	100

The effects of turkey strain on amnion consumption and hatching behaviour of late-term embryos.

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Nutritional reserves of an egg are limited and are considered key factors for the success of the avian hatchlings. These reserves, the surrounding microenvironment along with gas exchange and consequently water vapour loss affect embryonic growth and development and consequently hatchling size (Tullett and Burton, 1982).

There is a positive correlation between egg weight, embryo weight and amnion volume. Embryonic development of turkey is defined and categorized in 46 stages (Bakst *et al*., 1997). The early stages of embryonic growth and development are similar in most, if not all, avian species. However, in late stages of embryonic development (day 20 of incubation to hatch), major differences in terms of growth trajectory and development rate, are detectable between strains of turkeys.

Egg weight is not correlated with residual yolk weight during late stages of incubation; the same pattern is seen with hatchling weight and residual yolk weight. However, it seems there is a positive correlation between amnion volume and yolk weight. Older flocks have higher percentage of amnion and bigger embryos compared to younger ages (WOL 15 *versus* WOL 7), which can be attributed to higher initial egg weight and potentially higher albumen volume.

As the embryo weight increases between 20 and 25 days the volume of the amnion declines, chiefly on days 23 and 24 of incubation when it is consumed by the embryo. The duration of each stage is under direct influence of environmental factors, principally temperature and humidity, and it can be modified (prolonged or shortened) by modification of these incubation factors.

This study shows that the pattern of development, especially during the late stages, differed between the Hybrid and BUTA turkey strains. The duration of developmental stages, embryo growth rate, the pattern of amnion consumption, the rate of yolk uptake, and the hatching behaviour or pattern all differed between the two strains. Similar differences were observed in different strains of broilers and between broiler and layer chickens (A.E. Ghane, unpublished data).

These could be due to potential differences in genetic material of the strains. The variation or differences within a flock or strain could be attributed to surrounding microenvironment or internal reserve as well as heredity and genetic differences. The optimal incubation profile differs for each strain and this can affect the hatch success and incubation duration.

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PCB mixture (Aroclor1254) disturbs chick embryo development

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Sensitivity of the organism to polychlorinated biphenyls (PCBs) is particularly high during embryogenesis since mechanisms of xenobiotic detoxification are not fully developed during this period. Taking into consideration this information it was interesting to examine the effect of PCBs mixture on chicken embryogenesis.

The effect of Aroclor 1254 (mixture of several PCBs) on chicken embryogenesis and hatching process was investigated. Aroclor 1254 was injected *in ovo* at the fourth day of embryogenesis at doses of 0 (control), 10, 100, 1,000 ng/egg ($n = 120$ eggs/group). The incubation was carried out under standard conditions. Course of hatching was recorded for each group. Hatched chickens were weighted and then chosen tissues were taken. Blood samples were collected from randomly selected one-day-old chickens ($n = 10$ from each group). Following blood sampling, the chicks were decapitated and their livers and hearts were weighed. Thyroid hormones: thyroxine (T_4) and triiodothyronine (T_3) concentrations in plasma samples were determined by RIA method.

In ovo injection of Aroclor 1254 did not affect the final rate of hatching. There was no influence of the PCB mixture on body weight and weight of the examined tissues: liver and heart. Aloclor 1254 injected at doses of 10 and 100 ng/egg did not change T_4 concentrations; however, the highest dose of the PCB mixture decreased level of this hormone by 37% ($P \leq 0.05$). Aroclor 1254 at a dose of 10 ng/egg decreased by 20% T_3 concentration while at a dose of 1,000 ng/egg elevated T_3 concentration by 17% ($P \leq 0.05$).

The data presented suggest that injection of PCBs mixture *in ovo* at the early stages of embryogenesis affects embryonic development and influences thyroid hormone concentration in blood plasma in one-day-old chickens.

Licopene supplementation improves the survival of turkey semen after liquid storage

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In turkey, liquid storage beyond 6 h or freezing of semen does not preserve the viability of semen at the level necessary for commercial use. Therefore, the improvement of long-term liquid storage procedures of turkey semen is important since the commercial production of this bird relies almost entirely on AI (Iaffaldano *et al*., 2005). A previous study has shown that the addition of lycopene to semen extenders improves the cold survival of chicken semen (Mangiagalli *et al*., 2007). A carotenoid, lycopene is the most abundant pigment found in tomatoes and some red fruits and is considered the most efficient free radical scavenger. Despite this finding the effects of lycopene supplemented extenders on the survivability of sperm cells after chilled storage have not been studied yet in the turkey.

The aim of this study was to determine the effects of the supplementation of increasing amount of lycopene on *in vitro* turkey semen quality after 48 h of liquid storage. Five pooled semen samples were collected by dorso-abdominal massage from mature Hybrid Large White toms reared in a conditioned poultry house. Each pool was divided into four sub samples: the first aliquot was undiluted, the others were diluted four-fold in BPSE containing 0, 0.05 or 0.1 mg mL^{-1} of lycopene and kept at 5°C for 48 h on an orbital shaker at 150 rpm. Sperm motility (Accudenz® swim-down test), viability (SyBr-Propidium Iodide staining), osmotic resistance (Hyposmotic-water test), DNA integrity (acridine orange test) and lipid peroxidation (as malonaldehyde production) were evaluated on both fresh and stored semen**.** Data were compared with a one-way ANOVA followed by Duncan's comparison test (SPSS 14.0, 2005 version).

The semen quality and lipid peroxidation levels of fresh and chilled semen in absence or in presence of lycopene are shown in Table 1. Generally the semen quality after liquid storage worsened. However, the highest dose of lycopene recorded similar values for both viability and osmotic-resistance to those of fresh semen and significantly better than the values obtained in chilled semen without lycopene ($P < 0.05$). On the contrary, motility and DNA integrity were not affected by supplementation of lycopene in the extender. Lipid peroxidation was significantly higher after cold storage in all the treatments ($P < 0.05$), however spermatozoa chilled in lycopene enriched extenders showed significantly lower malonaldehyde levels than those chilled without lycopene.

In conclusion, the presence of lycopene in the extender improved the survival of turkey semen after liquid-storage. The beneficial effects of lycopene observed here could be related to its capacity to diminish sperm lipid peroxidation during refrigeration.

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Table 1 Qualitative parameters and MDA values (mean ± SEM) of turkey semen in freshly collected pooled samples and after their refrigeration in extenders without and with different amounts (mg mL^{-1} extender) of lycopene ($n = 5$)

Semen treatment	Sperm variable				
	Motility (Abs_{550})	Viability (%)	Osmotic resistance (%)	DNA integrity (%)	MDA (nmol/ $sperm^8$)
Fresh undiluted	0.37 ± 0.01[a]	84.48 ± 0.45[a]	74.61 ± 2.27[a]	98.90 ± 0.49[a]	0.45 ± 0.03[d]
Chilled in BPSE + lycopene 0	0.25 ± 0.01[b]	62.78 ± 3.92[c]	61.47 ± 2.26[b]	88.79 ± 2.45[b]	1.34 ± 0.04[a]
Chilled in BPSE + lycopene 0.05	0.28 ± 0.03[b]	72.82 ± 3.68[b]	62.66 ± 1.35[b]	91.27 ± 4.36[b]	1.07 ± 0.10[b]
Chilled in BPSE + lycopene 0.1	0.29 ± 0.01[b]	76.75 ± 2.11[ab]	68.89 ± 3.08[a]	92.04 ± 1.55[b]	0.95 ± 0.12[bc]

The effect of *in ovo* ascorbic acid and glucose injection in broiler breeder eggs on hatchability and chick weight

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The purpose of the study was to determine the effects of *in ovo* injection of ascorbic acid (AA) and glucose on the hatchability and chick weight of broiler eggs. AA was injected into broiler eggs to reduce stress experienced by the embryo associated due to the increase in metabolic heat production and the glucose was injected as a supplemental energy source. In Experiment 1, eggs were candled on the 13th day of incubation and live embryos were subjected to the following treatments, using the Embrex *in ovo* injection system: (1) uninjected (controls); (2) eggs injected with 0.5 mL sterile saline solution; and (3) eggs injected with 0.5 mL of saline solution containing 1, 3, 5 or 7 mg of AA per egg. In experiment (2), eggs were candled on the 18th day of incubation and live embryos were subjected to the following treatments, using the Embrex *in ovo* injection system: (1) uninjected (controls); (2) eggs injected with 0.5 mL deionized sterile water; and (3) eggs injected with 0.5 mL of deionized sterile water containing 5, 10 or 15 mg of glucose. The effect of AA injection on the hatchability of fertile eggs was found to be significant ($P < 0.01$). The highest hatchability was obtained from the group treated with AA at 3 mg concentration. No effect of glucose injection was determined on the hatchabilty and chick weight.

Influence of incubation temperature on mitochondrial activity in the breast and the leg muscle of broiler embryo

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Avian embryos are an excellent tool for the analysis of molecular mechanisms during embryogenesis (Scaal and Christ, 2004). During embryonic day (ED) 3 to 8, embryonic muscle development is characterized by the formation of primary myotubes. Hammond *et al*. (2007) showed that higher incubation temperature between ED 4 and 7 increases the muscle mass. Maltby *et al*. (2004) showed that an increased temperature between ED 5 to 8 affects myogenesis by increasing the total number of nuclei of the *M. semitendinosus*. In the present study, the impact of higher and lower incubation temperatures (± 1 °C) during two important periods of embryonic myogenesis (ED 7–10; ED 10–13) on embryo weight and mitochondrial respiratory activity (MRA) of permeabilised muscle fibres collected from the breast and the leg muscles were analysed.

In the first study, eggs of a commercial broiler line were incubated at 37.8 °C in a commercial incubator up to ED 7 and ED 10. At ED 7 and 10 each, half of the fertilised eggs were transferred to a second incubator of identical construction and were incubated at a higher (38.8 °C, Group H) temperature. After a further 3 days of incubation (ED 10, ED 13, respectively) the embryos were prepared. Each embryo was weighted and breast and leg muscle samples were removed for MRA analysis. In the second study the procedure was the same except the eggs were incubated at lower temperatures (36.8 °C, Group L) between ED 7–10 and ED 10–13. Analysis of MRA was performed as described by Janisch *et al*. (2012) by determining the state-3-respiration rates (in pmol $min^{-1}mg^{-1}$) with the substrates pyruvate/malate (Pyr/Mal). The data were statistically analysed (ANOVA) using the factors muscle type (leg, breast), treatment period (ED 7–10, ED 10–13) and incubation group (Group H, Group L). Embryos collected at ED 13 were generally heavier ($P<0.05$) in comparison to those of ED 10. However, no effect of the incubation group on the embryo weights could be determined at both collection days. The MRA values of the leg muscle fibres were significantly ($P<0.05$) higher at ED 10 and ED 13 in comparison to the breast muscles. The mitochondrial respiration values

of the ED 13-muscle samples were generally higher ($P < 0.05$) than the results at ED 10, independent of the muscle type and the incubation group. Considering the influence of the incubation group, the MRA results of the leg and breast muscle fibres, collected from Group H- embryos at ED 10 and ED 13, were significantly ($P < 0.05$) higher in comparison to the embryos, incubated at lower temperatures (Group L) between ED 7–10 and 10–13. The results indicate not only an influence of the muscle type (breast, leg) and embryo age (ED 10, ED 13), but also a positive effect of a higher incubation temperature between ED 7–10 and ED 10–13 on the mitochondrial respiratory activity. The effect of the muscle type might be related to structural differences and associated variation of the mitochondrial content, whereas the impact of the age and the incubation temperature on the MRA will be investigated in further studies using histological (muscle structure) and biochemical experiments.

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Effect of ascorbic acid *in ovo* administration on thyroid hormone concentration in blood plasma of chicken embryo exposed to hyperthermia during hatching

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The aim of this study was to investigate the effect of ascorbic acid (AA) *in ovo* administration on thyroid hormone concentration in blood of a chicken embryo exposed to hyperthermia during hatching period.

Hatching eggs of broiler chicken (Ross 308 line; $n = 360$) were *in ovo* injected with AA at a dose of 0 (control) or 2.5 mg/egg on the 17th day of incubation (E17). Next, the eggs from each group were divided into two equal parts that were incubated in standard (T 37.2°C, RH 55-65%) or hyperthermic condition (T 38.5°C, RH 55-65%). Blood samples ($n = 10$) were collected from embryos on embryonic day E18, during internal (IP) and external pipping (EP) and from new hatched chicks (D1). Thyroid hormones: thyroxine (T4), triiodothyronine (T3) and reverse triiodothyronine (rT3) concentrations in plasma samples were determined by RIA method.

The concentration of T4 in blood plasma of chicken embryos rapidly increased from 5.2 ± 1.80 ng mL^{-1}at E18 to 23.8 ± 2.15 during IP ($P \leq 0.05$) and gradually decreased to 9.9 ± 1.64 ng mL^{-1} at D1 ($P \leq 0.05$). AA *in ovo* administration increased the concentration of T4 at the EP by 32% compared to controls. Exposure of embryos to hyperthermia caused an increase in T4 concentration in embryonic blood plasma at E18 and EP by 64% and 33.7% in comparison with control group, respectively ($P \leq 0.05$). However, AA *in ovo* administration maintained T4 concentrations on a similar level as in the control embryos. T3 concentration in plasma of chicken embryos increased from 1.9 ± 0.52 ng mL^{-1} at E18 to 3.8 ± 0.47 at IP ($P \leq 0.05$), and subsequently gradually decreased to 1.5 ± 0.43 ng mL^{-1} at D1 ($P \leq 0.05$). AA administration increased levels of this hormone at the E18, IP and EP, by 67% ($P \leq 0.05$), 15% ($P > 0.05$) and 27.8% ($P \leq 0.05$), respectively. Overheating of embryos caused 2.8-fold increase in T3 concentrations in blood plasma at the E18 ($P \leq 0.05$). This phenomenon was significantly weakened by AA. The concentration of rT3 in hatching chickens was the highest at E18 (195 ± 334 pg mL^{-1}) and rapidly decreased at IP (7.8 times, $P \leq 0.05$). The level of this hormone at E18 in blood plasma of embryos treated with AA was 34 ± 16 pg mL^{-1} ($P \leq 0.05$) and remained at a similar level until their hatching. Exposure of embryos to hyperthermia resulted in a gradual decrease in rT3 concentration in blood plasma from 113 ± 11 pg mL^{-1} at E18 to 13 ± 2.5 pg mL^{-1} at D1. Administration of AA into hyperthermic embryos resulted in maintaining the level of rT3 at the level of 33–67 pg mL^{-1} between E18 and EP.

In conclusion, hyperthermia during hatching disturbs a thyroid hormone homeostasis in chick embryos. *In ovo* administration of ascorbic acid is able to reduce these disruptions by regulation of metabolic process in hatching chicks.

Using thermography to monitoring of development of thermoregulation of chick embryo (Gallus gallus)

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Avian embryo metabolism gradually increases during embryogenesis and the heat produced by the embryo needs to be lost from the egg. Egg shell temperature (EST) is considered to be a good indicator of incubation temperature. EST is normally measured using of a thermistor or a pyrometer and rarely thermal imaging camera. This study tests the usefulness of thermography as a method to monitor the development of chick embryo's thermoregulation.

The study was conducted on 600 hatching eggs of chicken broiler line Ross 308. The eggs were incubated in the setter S576 Petersime® in air temperature (T) 37.8°C and relative humidity (RH) 49-50%. Thermographic measurements of eggshell surface temperature (EST) was performed using a thermal imaging camera ThermoVision A20-resolution 120×160 pixel detector. For statistical analysis of each egg the highest EST values measured was used. Subsequent sessions were measured in following days of incubation: E1, E3, E4, E7, E8, E9, E11, E12, E13, E16 and E19. Trays with eggs were removed from the incubator and placed at T = 22.4 ° C, RH = 75%, and registration of EST was performed: (1) immediately after removing the eggs from the incubator, (2) after 15 min, (3) after 30 min. and difference between eggshell temperature in incubator and eggshell temperature of eggs cooling 15 ($DEST_{15}$) and 30 minutes ($DEST_{30}$) in following days of incubation was determined.

Thermographic measurements showed EST increase during subsequent stages of embryogenesis. The value of EST on E1 was 37.0±1.30ºC and increased during first 10 days of incubation about 0.1ºC at each day. During the following days of incubation the EST increased to 39.6±1.02ºC on E16, but on E19 significantly lowered to 38.0±0.78ºC.

The average EST decrease after 30 minutes of cooling inby 4ºC to 6°C; the most most rapid cooling occurring in the first 15 minutes. Analysing DEST in the following days of incubation can distinguish several phases related to the rate of heat loss by egg. The first falls on the initial hours of incubation, during which the contents of the eggs reach the right temperature for the activation of embryonic development. The second stage, between E2 and E6, was characterized by $DEST_{15}$ and $DEST_{30}$ on the level of about 3.5°C and by about 6°C, respectively. During third phase between E7 and E12, $DEST_{15}$ was below 3°C. However, between E13 and E17 (phase four) $DEST_{15}$ increased to 4.2°C, which can suggest highly sensitive to disruption of embryo thermal parameters. A small value of $DEST_{15}$ (only 0.8ºC) during hatching period (E18–E19) seems to indicate that at this stage of embryogenesis elements of thermoregulatory mechanism begin to function.

It was concluded that thermography would be useful method to monitoring of development of thermoregulation of chick embryo.

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Effects of temperature and CO_2 during the last 4 days of incubation on chick quality

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The goal of a hatchery is a high hatchability and a good chick quality, resulting in a good performance in later life. To optimize embryo development during incubation and thereby chick quality, incubation conditions need to be controlled and adapted to the requirements of the embryo. Temperature has been shown to be of major importance

for optimal embryo development and growth (French, 1994). A constant eggshell temperature (EST) of 37.8°C until day 18 of incubation has been shown to be the optimal temperature to gain the lowest embryo mortality, highest hatchability and optimal embryo development, expressed in a longer chick length, and higher yolk free body mass (YFBM) at day of hatch (Lourens *et al*., 2005).

However, little is known about the effect of applying different eggshell temperatures, simultaneously with different CO_2-levels from day 18 till 21 of incubation on embryo physiology, development, and chick quality. Therefore, it can be speculated that when eggshell temperatures lower or higher than 37.8°C in combination with different CO_2-levels are applied during the last 4 days of incubation, an effect can be found on embryo physiology, development, and subsequent chick quality.

The experiment was designed as a 3 x 2 experimental design with three EST (36.7, 37.8 or 38.9°C) and two CO_2-levels (2,000ppm or 10,000ppm) from day 18 till 21 of incubation**.** A total of 600 first grade hatching eggs (62–65 g; Ross 308 prime flock), were used. Until day 18, eggs were incubated at an eggshell temperature of 37.8°C in a HT-combi incubator with the capacity of 4,800 eggs (HatchTech BV, Veenendaal, the Netherlands). From day 18 until day 21 of incubation, three EST and 2 CO_2-levels were applied.

At internal pipping (IP), hatch, and 12 hours after hatch, different chick quality parameters were measured; chick weight, yolk weight, YFBM, and chick length. Chickens were not provided with food nor water and had continuous light. Results showed an interaction between EST and CO_2 for chick weight ($P<0.0001$). At an EST of 37.8°C, chick weight ($\Delta = +0.84$g), yolk weight ($\Delta = +0.68$g), YFBM ($\Delta = +0.15$g), and chick length ($\Delta = +0.27$cm) were higher at a CO_2-level of 10,000ppm compared to a CO_2-level of 2,000ppm. However, at an EST of 36.7 or 38.9°C, chick weight ($\Delta = -0.22$g), yolk weight ($\Delta = -0.08$g), YFBM ($\Delta = -0.14$g), and chick length ($\Delta = -0.22$cm) were lower at a CO_2-level of 10,000ppm compared to a CO_2-level of 2,000ppm. Furthermore, embryos incubated at an EST of 36.7°C compared to 37.8°C and 38.9°C had a higher chick weight (45.62g, *versus* 45.57 and 45.39g), lower yolk weight (6.64g, *versus* 6.81 and 6.82g), higher YFBM (38.97g, *versus* 38.75 and 38.57g), and longer chick length (19.63cm, *versus* 19.60 and 19.58g).

The higher chick quality gained by the reduced EST of 36.7°C may be related to the extended incubation time of 6 hours, which provided the embryo the possibility to convert more yolk nutrients into body mass. This is in agreement with lower yolk weight at low EST of 36.7°C compared to normal (37.8°C) and high EST (38.9°C). In conclusion, a low EST of 36.7°C compared to normal EST (37.8°C) and high EST (38.9°C) improves chick quality. Furthermore, the effect of CO_2-level on chick quality is dependent on the EST. The reason for this is unclear, but might be related to the differences in physiological characteristics which are currently under investigation.

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Hatchability of pheasant eggs fertilised with cryopreserved semen from dietary manipulated males

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It is well known that avian spermatozoa are sensitive to lipid peroxidation due to the kind of phospholipids that form their membrane. Consequently protection against peroxidation, especially during the freezing–thawing procedure is necessary to maintain the structural integrity of the spermatozoa.

The aim of the present investigation was to test *in vivo* the fertilising capacity of cryopreserved semen obtained from males fed on two diets differing for selenium/vitamin E ratio (Se/Vit E). A basal commercial feed (11.51 MJ kg^{-1} of M.E., 19% of C.P.) with 1% fish oil, was enriched with 40 mg vitamin E plus 0.1 mg selenomethionine per kg of feed or with 200 mg vitamin E plus 0.3 mg selenomethionine per kg of feed, thus to obtain 0.0025 and 0.0015 Se/Vit E, respectively. The pheasants were fed the diets for 1 month and then semen was collected from the males and processed for the freezing procedure in pellets as described by Castillo *et al*. (2011). 100μL

Table 1 Characteristics of fresh and thawed semen and the effect on semen characteristics of two different diets offered to two groups of male pheasants at one month before the ejaculates collection

	Dietary Se/Vit E	
	0.0025	0.0015
	mean ± SD	mean ± SD
Fresh semen		
pH	8.30 ± 0.17	8.30 ± 0.04
Sperm concentration (10*9/mL)	7.30 ± 0.69	8.45 ± 0.32
Dead spermatozoa (%)	18.92 ± 4.25	16.15 ± 3.22
Morphologically normal spermatozoa (%)	61.55 ± 7.28	58.73 ± 4.42
Mobility (A_{550nm})	0.298 ± 0.045	0.330 ± 0.034
Thawed semen		
Dead spermatozoa (%)	71.71^{A} ± 3.25	65.80^{B} ± 3.15
Morphologically normal spermatozoa (%)	10.90^{B} ± 1.50	18.67^{A} ± 0.98
Mobility (A_{550nm})	0.042^{B} ± 0.005	0.066^{A} ± 0.002

^{A,B} $P < 0.01$.

Table 2 Fertility and hatchability of pheasant eggs fertilised by dietary manipulated cryopreserved semen

	Dietary Se/Vit E	
	0.0025	0.0015
Set eggs (*n*)	81	85
Fertility (%)	33.33	25.88_{ns}
Hatchability on fertile eggs (%)	29.63	31.82_{ns}

ns Not significant.

semen pellets were stored in liquid nitrogen for one year and then thawed by a hotplate at 75 °C and used for artificial inseminations (AIs). The thawing procedure was performed as described by Marzoni *et al*. (2009).

Hatchability was evaluated on eggs laid by 10 female pheasants in a 19-day period. The females were divided into two groups according to the dietary semen group received. Each female received doses of 31 to $37x10^6$ live normal thawed spermatozoa. The first two AIs were performed on successive days and thereafter AI was carried out twice a week, for a total of six AIs. Sperm qualitative parameters were assessed as described by Marzoni *et al*. (2009). Data were subjected to analysis of variance, percentage data were arcsine transformed prior to analysis. Egg parameters expressed in percentages were compared by the χ^2 test.

Table 1 reports some qualitative parameters of fresh and thawed semen in relation to the dietary treatment. In fresh semen there were no significant differences for any of the parameters as previously observed by Marzoni *et al*. (2010). In the thawed semen, less dead, more normal and better performing cells were obtained in the 0.0015 Se/Vit E group. In contrast, results of the *in vivo* study (Table 2), showed no significant differences between the dietary semen groups. As egg production was the same between the two female groups assigned to test the cryopreserved semen, the environment within the oviduct of the female allowed the expression of some unknown male cell defects, such as, for example, a defective chromatin in sperm with microscopically normal morphology.

In conclusion, in this study more resistant and better performing cell *in vitro* was found in cryopreserved semen from males fed the diet supplemented with 200 mg Vitamin E plus 0.3 mg organic selenium per kg of feed.

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Practical implementation of short periods of incubation during egg storage

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At the IFRG meeting in 2011 we described four experiments which showed that short periods of incubation during storage (SPIDES) could give improvements in hatchability of longer-stored eggs. (Nicholson *et al,* 2011). Since then we have been investigating the effectiveness of the technique on a larger scale in Aviagen's commercial breeding stock hatcheries in the UK, USA, Hungary, India and New Zealand. A total of eight trials have been now been completed, covering a range of Aviagen GGP and GP lines and crosses.

The first commercial trial was run in New Zealand, using eggs laid by great-grandparent stock. The eggs were stored for 21 days, and given four x 4 hours heat treatments on days 5, 10, 15 and 18 of storage. All three lines responded similarly to the storage treatment, giving an average of 30 percentage point improvement in hatch of stored eggs.

A second trial in the UK looked at the impact of flock age, comparing results from flocks of 33 and 51 weeks of age. SPIDES (four treatments) improved the hatch of stored eggs in both groups, giving a 13 percentage point improvement in hatch of stored eggs.

Two trials in India over a total of eight Aviagen lines and crosses, giving 4 x 4-hour treatments for eggs stored for between 22 and 24 days, laid by hens across a range of flock ages, gave an average improvement in hatch of 9.5%.

Trials in Hungary looked at shorter storage and fewer treatments. Eggs stored for 18 days and heat treated three times hatched 19% points better after SPIDES treatment. Eggs stored for 8–9 or 13–14 days and treated once at around 4 days were improved by 6 and 5% points respectively.

Unfortunately the trials in India, Hungary and the USA did not include a positive control, so it is not possible to tell if the amount of improvement possible was limited because the hatch loss over storage was lower.

Finally, two trials in the USA, stored for 11–13 or 10–14 days and treated only once, at 3–7 days of storage showed no improvement in either trial, although the chick quality was slightly better. The reason for the lack of response is not known, but is thought to be due to differences in egg collection and storage which slow the cooling of the eggs after collection, and may be allowing the embryo to develop beyond a stage where SPIDES is useful. This is being investigated at the time of writing.

Practical implementation of SPIDES has shown that in commercial parent stock hatcheries improved hatchability can be achieved by using SPIDES on eggs which are destined for longer storage.

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Effects of high incubation temperature on small intestine development of broiler embryos: evaluation of histochemical, immunohistochemical and histological findings of two commercial strains

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Today's broiler chicken spend up to 30–40% of its total life inside the egg and anything affecting positively or negatively the growth and development during embryonic period can have a marked impact on posthatch performance of the chicken (Hulet *et al*., 2007; Barri *et al*., 2011). Embryonic development of the small intestine has a major role in determining the developmental potential of the hatched chick. Thus, comparison the intestinal development of the chickens from different broiler strains experimentally exposed to heat stress may provide important clues in predicting growth and production traits. In this study, the effects of high incubation temperature on the embryonic development and Hsp70 expression of the small intestine in Ross 308 and Hybro strains were investigated and compared.

Table 1 Hsp70 immunoreactivity of small intestine epithelium at different incubation period of the groups

Sampling periods	Part of small intestine	Groups			
		Hybro control (Mean ± SE)	Hybro heat-stress(Mean ± SE)	Ross308 control(Mean ± SE)	Ross308 heat-stress(Mean ± SE)
11th day of incubation	Duodenum	3.00 ± 0.274	3.40 ± 0.245	2.60 ± 0.187	3.20 ± 0.200
	Jejunum	2.80 ± 0.123	3.20 ± 0.200	2.70 ± 0.123	3.20 ± 0.339
	Ileum	2.80 ± 0.339	3.10 ± 0.367	2.70 ± 0.123	3.10 ± 0.339
13th day of incubation	Duodenum	2.70 ± 0.255	2.70 ± 0.123	2.50 ± 0.158	3.10 ± 0.400
	Jejunum	2.60 ± 0.187	2.80 ± 0.339	2.50 ± 0.158	3.00 ± 0.274
	Ileum	2.70 ± 0.123	2.80 ± 0.200	2.60 ± 0.100	2.70 ± 0.123
15th day of incubation	Duodenum	2.20 ± 0.300	2.60 ± 0.187	2.30 ± 0.123	2.40 ± 0.292
	Jejunum	1.82 ± 0.472	2.10 ± 0.100	2.00 ± 0.158	1.70 ± 0.123
	Ileum	2.40 ± 0.187	2.50 ± 0.158	2.20 ± 0.255	2.50 ± 0.158
18th day of incubation	Duodenum	2.00 ± 0.158	2.70 ± 0.374	2.00 ± 0.158	2.50 ± 0.224
	Jejunum	2.10 ± 0.292	2.30 ± 0.123	2.10 ± 0.292	2.20 ± 0.255
	Ileum	2.30 ± 0.200	2.70 ± 0.255	2.20 ± 0.125	2.40 ± 0.187
20th day of incubation	Duodenum	2.30 ± 0.464^{ab}	3.20 ± 0.339^{a}	1.70 ± 0.200^{b}	3.20 ± 0.200^{a}
	Jejunum	2.20 ± 0.374	3.10 ± 0.367	1.70 ± 0.464	3.10 ± 0.367
	Ileum	2.20 ± 0.374^{b}	3.10 ± 0.100^{b}	2.00 ± 0.474^{b}	3.40 ± 0.245^{a}
21st day of incubation	Duodenum	1.70 ± 0.436^{bc}	3.30 ± 0.200^{a}	1.10 ± 0.100^{c}	3.00 ± 0.274^{ab}
	Jejunum	1.50 ± 0.316^{b}	2.60 ± 0.187^{a}	1.10 ± 0.100^{c}	2.50 ± 0.224^{ab}
	Ileum	1.60 ± 0.400^{ab}	2.50 ± 0.224^{a}	1.20 ± 0.200^{b}	2.80 ± 0.374^{a}

[a-c] Means followed by different letters in the same line are significantly different ($P < 0.05$).

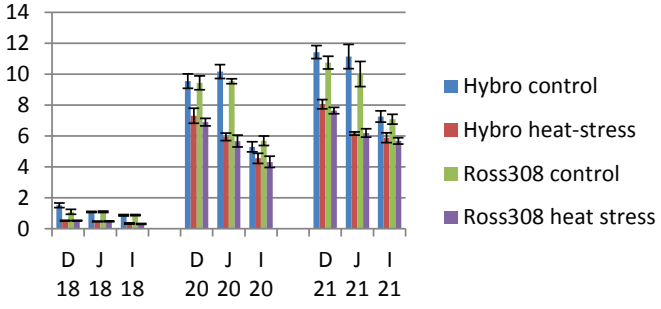

Figure 1 Relative ALP activity of small intestine at different incubation period of the groups (%). Results are presented as means, and bars are SE. The differences between control and heat stress groups of both strains are significant ($P < 0.05$).

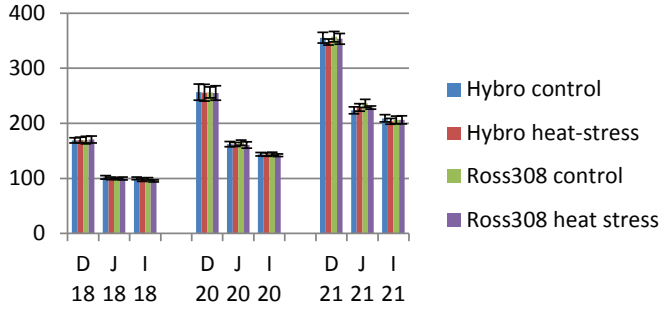

Figure 2. Mean villus height of duodenal, jejunal and ileal villi at different incubation period of the groups. Results are means and bars are SE. The differences between control and heat stress groups of both strains are not significant ($P > 0.05$).

In the experiment, 500 fertile eggs were used. The eggs of each strain were divided into two groups as control and heat-stress groups. The control eggs were maintained under optimal conditions (37.8°C, 50-65% relative humidity) during the whole incubation period; whereas heat stressed eggs were maintained under the optimal conditions until the 10th day of incubation and then continuously exposed to high temperature (38,8oC). Tissue samples were taken from 10 embryos of each group at 11th, 13th, 15th, 18th, 20th and 21st days of incubation. After processing, tissue sections were stained with Crossmon's trichrome and Hsp70 were detected using immunohistochemical methods. ALP activity was demonstrated in frozen sections. ALP activity, which is considered to be a maturation marker for enterocyte, significantly ($P < 0.05$) declined in the heat-stress group, when compared to that of the control group of each strain (Figure 1). Hsp70 immunoreactivity changed in a similar manner in both control and stressed groups until 18th day of incubation. However, it significantly increased in different segments of the intestines of heat stress groups (Table 1). Hsp70 expression in the small intestine was affected by incubation temperature in an age-dependent manner. Intestinal histology and villus heights were quite similar in both control and heat-stress groups of investigated strains (Figure 2). In this study high incubation temperature affected two strains in a quite similar manner except for minor differences. These results may contribute to the understanding of mechanisms by which embryonic development can be affected by high temperature.

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Histochemical and histological evaluations of the effects of high incubation temperature on embryonic development of thymus and bursa of Fabricius in broiler chickens

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For most poultry species, optimum incubation temperature ranges have been reported between 37 and 38°C, and it should not vary by more than 0.3°C since small deviations from this optimum can have a major impact on hatching success and embryo development (Wilson, 1991). Avian embryos are sensitive to both lower and higher incubation temperatures: lower incubation temperatures depresses and higher incubation temperatures accelerates the growth and development (French, 1997).

Prehatching stress in chickens might cause deleterious effects, similar to those of prenatal stres observed in mammals (Lay and Wilson, 2002). Among the most pronounced effects of prenatal stress in mammals is the elevation of glucocorticoid hormones (Weinstock, 1997). Glucocorticoids, also known as stress hormones, have profound destructive effects on the lymphoid tissues and immune responses. Lymphocytolytic effects of experimentally administered cortisone occur within hours, and lead to a dose-dependent thymus weight loss.

Because the thymus and bursa of Fabricius play crucial roles in enzymatic maturation and acquisition of immunological competence of T- and B-lymphocytes (Rudrappa and Humphrey, 2007), any disturbances in the embryonic development of tymus and bursa of Fabricius might result in significant deficiencies of the immune system functions of the chicken. In this study, the effects of experimentally induced heat-stress on the embryonic development of bursa of Fabricius and thymus of the chicken were investigated by means of histological and enzyme histochemical methods.

In the experiments, 250 fertile eggs of the Ross 308 broiler strain were divided into two groups. The control eggs were

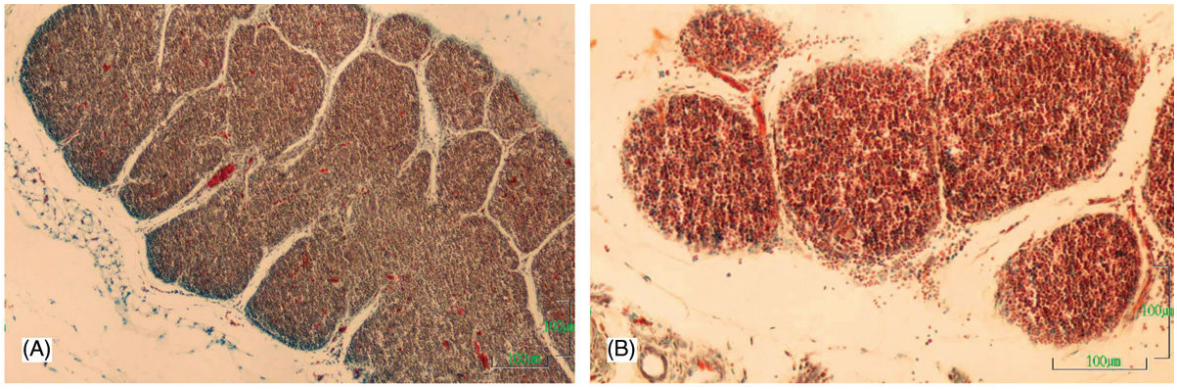

Figure 1 Normally developing thymic lobuli in thymic section of control group (A), and poorly developed lobuli of heat-stress group (B) at day 15 of incubation. Trichrome. Bar: 100μm.

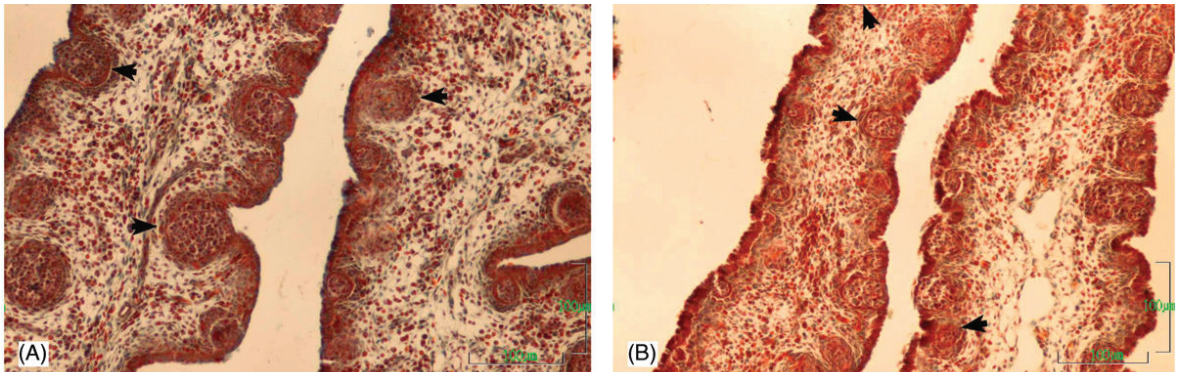

Figure 2 (A) A section from bursa of Fabricius of control group, at day 15 of incubation. Normally developing bursal follicles (arrow heads) are seen. Trichrome. Bar:100μm. (B) A section from bursa of Fabricius of heat-stress group, at day 15 of incubation.Poorly developing bursal follicles (arrow heads) are clearly seen. Trichrome. Bar: 100μm.

maintained under optimal conditions (37.8°C and 65±2% relative humidity, RH) during the whole incubation period. Heat stressed eggs were maintained under normal conditions (37.8°C and 65±2% RH) until the 10th day of incubation and then exposed continuously (24 h per day) to high temperature (38.8°C and 65±2% RH). Blood and tissue samples were taken from 10 animals of each group at day 13, 15, 18 and 21 of incubation and at days 2, 4 and 7 posthatch. Tissue samples were processed for enzyme histochemical methods in addition to routine histological techniques.

The results revealed that egg temperatures were higher than incubator air temperature. Long-term heat-stress (40.1–40.6 °C egg temperature) retarded development of thymus (Figure 1). and bursa of Fabricius (Figure 2). Peripheral blood ACP-ase and ANAE-positive lymphocyte levels of heat-stressed animals were lower than in the controls.

These results give some morphological evidence for immunosuppression induced by high temperature exposure during the embryonic development. Temperature distribution and air circulation in incubator should be questioned in the case of lower broiler flock immunity.

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The effect of antioxidants on frozen-thawed chicken sperm

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The objective of this study was to investigate the effect of cysteine and superoxide dismutase on quality of frozen-thawed chicken semen. Pooled semen samples collected from 15 Greenlegged Partridge roosters were divided into three aliquots and extended at the 1:2 ratio with: EK diluent (control), EK diluent supplemented with 5 mM N-acetyl-L-cysteine (NAC) and EK with 200 U mL^{-1} superoxide dismutase (SOD). Diluted samples were subjected to cryopreservation using the "pellet" method and dimethylacetamide as a cryoprotectant.

Post-thaw sperm motility characteristics: total motility (MOT), progressive motility (PMOT), average path velocity (VAP), curvilinear velocity (VCL), straight line velocity (VSL), straightness (STR), linearity (LIN), subpopulation of rapid cells (RAPID) were determined by CASA (IVOS system ver. 12.2l, Hamilton Thorne Biosciences, MA, USA); sperm plasma membrane integrity was assessed with dual fluorescent probes, SYBR-14 and propidium iodide (Live/Dead Sperm Viability Kit); acrosomal damages (with the use of lectin PNA-Alexa Fluor from *Arachis hypogaea*); the mitochondrial activity was estimated using JC-1; Annexin-V/PI was used to assess the apoptotic changes and C_{11}-BODIPY$^{581/591}$ stain was used to assess lipid peroxidation within spermatozoa membranes.

In the thawed semen the MOT was significantly higher ($P<0.01$) in the NAC group (15.70±4.3%) and SOD group (12.72±2.8%), compared to the control group (8.20±2.1%). Progressive motility was higher ($P<0.01$) in the NAC extender (2.32±1.2%) than in the SOD (1.68±0.9%) and control (1.10±0.7%) diluents. The percentage of RAPID cells was higher ($P<0.01$) in the NAC group (3.52±1.4%) and in SOD (2.63±1.1%) than in the control (1.97±1.2%). The addition of antioxidants did not affect the VAP, VSL, VCL, STR. The NAC and SOD addition resulted in a significantly ($P<0.05$) higher percentage of viable sperm (38.80±4.5% and 39.99±4.4%, respectively) following cryopreservation, than the control (31.77±2.0%). No significant differences were observed in sperm acrosome integrity among groups. Percentage of sperm with high mitochondrial activity was higher ($P<0.05$) with NAC (65.58±3.9%), compared to the control (60.46±5.4%). After freezing-thawing significantly lower percentage of live sperm with apoptosis in NAC group (11.18±3.3%; $P<0.01$) and SOD group (12.51±2.8%; $P<0.05$), compared to control (19.52±6.6%) was observed and significantly lower ($P<0.05$) percentage of live sperm with lipid peroxidation in the samples with the addition of SOD (1.01±0.7%), in relation to control samples (1.93±0.8%).

In conclusion, N-acetyl-L-cysteine or superoxide dismutase supplementation of EK diluents improved motility, progressive motility, viability and mitochondrial activity of frozen-thawed chicken sperm. Moreover, the antioxidants provided a cryoprotective effect, suppressing apoptotic changes and lipid peroxidation in sperm membranes.

Influence of radio electromagnetic fields on the chicken hatching

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Over the last decades, a remarkable increased level of the electromagnetic field (EMF) in the environment has characterized modern societies. The aim of this study was to investigate the influence of EMFs on chicken embryo hatchability.

Hatching eggs of Ross 308 line (n = 180) were used in the experiment. The eggs were randomly divided into three equal groups and incubated under standard conditions in a laboratory incubator. Group I was incubated in control conditions, *i.e.* in the incubator without an EM field generator, Group II (sham group) was set in the incubator with the EM field generator switched off, Group III (experimental group) were chicken embryos subjected to exposure to magnetic fields (900 Hz) 10 times per 4 minutes of every day. The laboratory radio frequency generator was composed of three main entities: steering, generating and emitting. The generating component was made using laboratory equipment: continuous power emission without any modulation was possible with the maximum power output of 330 mW. Simultaneously the generator can emit one selected frequency from two GSM frequency bandwidths.

Hatch times were observed from 430h of incubation. Chicks in the control and sham groups took 34 hours to hatch with the peak of hatching taking place between 482 and 490h. Group III eggs hatched approximately 24h earlier than the controls and between 459 and 465h 80% of the chicks had hatched. Group III chicks hatched 4.6% below the hatch of Group I and II eggs. While in the sham and control groups, the highest numbers of dead chicks were observed on the 19th and 20th days of incubation, the highest death rate in the experimental group was observed on the 5th and 21st days of incubation. Dead embryos from Group III eggs showed an increased incidence of ruptured blood vessels, sub-cutaneous haemorrhaging and hyperaemia of internal organs.

Pre-natal thermal manipulation affects muscle development in broiler chickens

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A previous study conducted in our laboratory on meat type chickens showed that intermitted thermal manipulations (TMs) of 39.5°C for 12h, during embryonic (E) days E7 to E16 improved thermoregulative parameters in the embryo and posthatch. In addition, TMs at this temperature for 3h and 6h daily, between E16 to E18, increased proliferation of embryonic muscle cells (myoblasts) resulted in enhanced muscle growth posthatch. The aim of this study was to investigate the effect of intermitted TM during the time window of foetal myoblast proliferation (E7 to E16) on breast muscle development during embryogenesis and after hatch.

Muscle myoblasts were prepared from breast muscle of broiler embryos from E10 till hatch and on days 3, 6, 9 and 13 posthatch. The number of myoblasts per gram of breast muscle was determined and the proliferation ability of the myoblast was examined by DNA thymidine incorporation in culture. The results show that TM elevated the number of myoblasts in the embryo breast muscle from E10 until E18. This increase in myoblast number per gram of breast muscle was more pronounced from E10 until E15 where the myoblast number was 20–50% higher in the TM embryos. The positive effect of TM on myoblast number sustained posthatch too. The number of myoblasts in the breast muscle of TM chicks on the third day after hatch, in which myoblast number reach a peak, was 40% higher compared to the control. This increase in myoblast number was a result of an increase in the myoblast ability to proliferate. Thymidine incorporation into the DNA of in-cultured myoblasts derived from TM embryos was significantly higher compared to control till E15. After hatch, it was higher by 31 and 25% on days 3

and 6, respectively, for the TM chicks. Simultaneously, the percent of breast muscle from body weight was significantly higher in the TM embryos from E10 till hatch, and in the TM chicks during the first 2 weeks after hatch.

This study demonstrates that TM during the foetal myoblasts appearance increases the myoblast ability to proliferate and enhance cell number in the breast muscle tissue of embryos and of chicks during the first days posthatch. This may result in higher myonuclei number in the muscle tissue, suggesting increased growth potential of the muscle posthatch. These results shed light on previous findings demonstrating a significant increase in breast muscle relative weight on day 35 which was accompanied by larger myofiber diameter in the TM chickens.

Effect of oxygen supplementation in the hatcher at high altitude on the incubation results of broiler eggs laid at low altitude

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The aim of this study was to investigate the effect of high altitude on the final period (plateau

and paranatal stages) of embryonic development of eggs incubated under hypoxic conditions. We tested the effects of oxygen supplementation in the hatcher, and we measured the incubation results of broiler eggs laid and incubated at high altitude in comparison with these measurements for broiler eggs laid and incubated at low altitude. A total of 2250 eggs were obtained from broiler breeder parent stock at 50 weeks of age at low altitude (100 m). In the setter, each tray contained 150 eggs and five trays (750 eggs) were used for each treatment groups. Initial incubation conditions for each treatment were identical and followed normal commercial practice. The first group was set in an incubator and hatcher cabinet at low altitude of 100 m with an average barometric pressure of 754 ± 10 mm Hg. The percentage of oxygen was measured to be 20.9 + 0.5 % during the experiment and the first group did not receive any supplemental oxygen between days 18–21 incubation. The second and third groups were set in same incubator at high altitude of 1100 m and an average barometric pressure of 674 ± 10 mm Hg for 18 days of incubation. On transfer day, these eggs were set in two different hatcher cabinets. The one hatcher cabinet was supplemented with oxygen between days 18 and 21 incubation. Oxygen concentration within the hatcher cabinet was regulated to 23 ± 0.5 % O_2 with a flow rate of approximately 15 L min^{-1}. The other cabinet did not receive any oxygen. High altitude affected relative egg weight loss and early embryonic mortality. The hatchability of fertile eggs was lower at high than at low altitude. Oxygen supplementation into the hatcher cabinet during the last stage of incubation decreased late embryonic mortality ratio and improved survival rates of embryos incubated at high altitude. Eggs incubated at low altitude had a higher hatched chick weight and relative chick weight than those incubated at high altitude. Hatched chick weight and relative chick weight did not change with oxygen supplementation at high altitude. Therefore, providing supplemental oxygen might be a good way of improving hatchability and chick quality at high altitude.

Effects of canthaxanthin and 25-hidroxicolecalciferol on the reproductive performance of broiler breeders from 25 to 52 weeks of age

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Chick embryo development is associated with an accumulation of polyunsaturated fatty acids in lipid tissues making them susceptible to lipid peroxidation. It has been accepted that canthaxanthin has high antioxidant capacity. Research findings have indicated that feeding 25(OH)D3 (25-hydroxycholecalciferol), to poultry breeders, broilers and layers increased performance and health. Therefore, one experiment was conducted with the objective to evaluate the effects on performance and hatchery parameters of a product called Rovimix®

MaxiChick (association of 6 ppm of canthaxanthin and 69 mcg kg^{-1} feed of 25(OH)D3) fed to broiler breeders. Diet treatments were fed to 264 females and 24 males COBB 500 broiler breeders between 25 and 52 weeks of age. The diets were based on corn and soybean meal. The two treatments were: control diet and control diet with Rovimix® MaxiChick. The parameters evaluated were body weight, laying rate, egg weight, specific gravity, percentage of albumen, yolk and shell, yolk colour. Other parameters evaluated were the hatching rate, hatching of fertile eggs, fertility, embryo mortality, chick's weight and percentage of chicks of low quality. The laying rate was measured by six daily collections during the experimental period. Eggs collected once a week were used for all the other analyses. Non-hatched eggs were submitted to embryo diagnostics.

The addition of MaxiChick in diets of broiler breeders did not adversely affect the productive performance of birds nor the quality of eggs. Broiler breeders fed with MaxiChick had the highest deposition of carotenoids in the yolk, and this diet also increased the hatchability ($P < 0.0075$) from 87.15 to 89.67%, the hatchability of fertile eggs ($P < 0.0237$) was increased from 91.63 to 92.60% and the percentage of early embryo mortality (first 48 hours of incubation) ($P < 0.0178$) reduced from 1.42 to 1.04% during the total period evaluated. The number of contaminated eggs, average weight of chicks and the percentage of low chicks and fertility were not affected by the addition of products. In conclusion MaxiChick improves most of the reproductive parameters evaluated in this trial.

Effect of different concentrations of vitamin A on the chick broiler embryo development

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This study investigated the effects on broiler embryo development of injecting fresh eggs with four concentrations of vitamin A, in the form of vitamin A palmate dissolved in sterilised corn oil. Four concentrations of vitamin A, 3500, 4200, 6000 and 6800 I.U. were injected into the air cell of the fresh eggs, 90 eggs per treatment. Eggs were incubated at 38ºC for 21 days and the embryo morphological abnormalities were studied at 3, 5, 7, 9, 11, 13 and 21 days of incubation.

Increasing vitamin A concentration resulted in an increase in embryo mortality from 41.7% to 69.6%, with the LD50 at 4200 I.U. Increasing concentrations of vitamin A reduced embryo weight and length. The morphological examination of embryos at different ages shows increased abnormalities of the eye (anophthalmia, microphthalmia and different eyes sizes); of the head and brain (anencephaly, exencephaly and undifferentiated brain seen at 3 days of incubation); of the beak; of the heart; of the limbs (crooked limbs and toes); of the thorax and abdomen (omphalocele).

Assessment of different day-old chick quality measurements as a breeding trait in layer type chicken

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In recent years, chick quality has become a research topic of interest, with the aim of finding a clear and objective definition of chick quality in order to be able to hatch and detect chicks with good growth potential at one-day of age (Willemsen *et al*., 2008). However chick quality is still difficult to quantify, and interpretation and comparison of the results is demanding.

The aim of this study is to record different quality characteristics of the chick and evaluate their potential use as a selection trait for improving chick quality. For this purpose, chicks from three hatches of a pure-bred female line of an LSL commercial breeding programme were individually scored for different qualitative traits on day 0 in the hatchery: body weight (BW0) and chick

Table 1 Estimates of heritability (on the diagonal) for chick quality traits and the genetic correlations (above the diagonal)

BW0	CL	CA	NA	NM	RH	CAP	CB	BW1
0.55	+0.87	+0.12	+0.36	-0.05	+0.21	+0.18	-0.41	+0.81
	0.80	+0.71	+0.64	+0.14	+0.27	+0.61	+0.63	+0.65
		0.16	+0.44	+0.02	+0.60	+0.48	+0.51	+0.45
			0.12	+0.48	+0.51	+0.91	+0.58	+0.52
				0.11	+0.16	+0.58	+0.36	+0.07
					0.12	+0.38	+0.15	+0.31
						0.11	+0.66	+0.28
							0.13	+0.08
								0.71

Table 2 Differences between the measurements of alive and dead chicks (mortality until end of rearing)

	Alive	Death
BW	40.4	40.5
CL	18.2	18.2
CA	8.0 [a]	7.3 [b]
NA	7.4	7.2
NM	8.1	8.1
RH	8.7	8.9
CAP	8.8	8.9
CB	8.7	8.5
Sum score	9.2	9.0

length (CL). Furthermore, an evaluation score (between 1 and 9, the higher the better) was given for the following traits: chick activity (CA), evaluation of the navel area (NA) and navel membrane (NM), presence of red dots in hooks or beak (RD), chick appearance (CAP), evaluation of the chick's belly (CB) and finally the sum note of the different scores was calculated (SN). Chick weight at one week of age was also recorded. In total, data from 3,454 chicks were available for the analysis. On average, there were 6.6 eggs set per dam and hatch, and the hatchability of eggs set was 81%.

As shown in Table 1, body weight at hatch and chick length had a high heritability and a high genetic correlation (rg = 0.87), whereas the phenotypic correlation was 0.56. The qualitative scores had heritabilities ranging between 0.11 and 0.16 and were positive correlated with each other. Besides body weight at hatch and chick length, especially interesting seems to be chick appearance and navel area, which had the higher occurrence of abnormalities and the higher heritabilities. They had also a high genetic correlation with body weight at one week of age.

According to Table 2 the difference in the activity score at hatch was significant ($P < 0.05$) between the chicks that died and those, who were alive at the end of the rearing with 18 weeks of age. Due to the fact that cumulative mortality in the first 18 weeks of rearing was low, (1.74 %, which means 60 from 3,454 day old chicks) it is not possible to make any clear conclusion.

We have shown that it is possible to select for better chick quality by taking different chick quality parameter into consideration. Since the measurements are very time consuming and the heritabilities quite low, the major improvements will have to come from optimisation of breeder flock, hatchery and brooding management.

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The effects of monochromatic lighting on embryonic development and hatching performance depending on egg shell colour

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In this study, the effects of monochromatic lighting on embryonic development and hatching performance depending on egg shell colour were investigated. The number of eggs used in the two separate experiments were 780 light brown (Ross-308 genotype) and 720 dark brown eggs (Hubbard Red JA genotype) respectively. The three treatment groups were as follows: Group 1, control group (no light); Group 2, green light (560 nm and WL range from 535 to 585 nm); Group 3, red light (670 nm, WL range from 640 to 690 nm). In the light treatments, an intensity of 0, 1 and 2 lux continuous illumination was provided during the first 18 days with LED lamps were placed on each side of the trays. The normal incubation scheme was used during the incubation period (0–18 days: 37.7 ± 0.1 °C and 60 ± 5 %RH; 19–21 days: 37.2 ± 0.1 °C and 75 ± 5 %RH). Hatching time was recorded every 3 h between 474 and 507 hours of incubation. After hatching, chick weights and chick lengths of 20 chicks from each group were recorded.

There was no effect of monochromatic lighting on chick weight, but it was found that both light colours significantly increased chick length. Red light and control groups (14.07% and 14.62%) show similar embryonic death rate, while the green light group had lower embryonic mortality rate (5.88%) than these groups in the first experiment. There was no difference between the examined hatching performance parameters of the treatment groups in the second experiment. In both experiments, the effects of monochromatic lighting on the hatching time were significant (χ^2, $P<0.05$), and also there were significant differences in hatching trends (Kendall's Tau-b 0.03).

The findings of this research carried out with two different light wavelengths and two different genotypes are remarkable for showing that monochromatic illumination has significant effects in the hatchery, on embryonic development as well as on hatching performance.

Imprinting of body functions by manipulation of incubation temperature and its long-lasting effect on hatchability, performance and health in poultry

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'Imprinting' describes a fundamental process of life, which occurs during 'critical periods' of embryonic or early postnatal development and has effects that last into adult life and possibly over generations. It is probably realised by neuronal plasticity, as well as by a lasting, environment-induced (epigenetic) modification of the genome. A 'critical period' for the 'imprinting' of body functions is the development of feedback mechanisms, which occurs during the perinatal period. During this period the actual level at which physiological parameters are active may pre-determine a life-long 'set point' (or 'set ranges') for the respective regulatory system.

In poultry embryos, during the final days before hatching the thermoregulatory system develops feedback mechanisms. During this phase, the thermoregulatory system can be imprinted by manipulation of incubation temperature. The effect of this manipulation strongly depends on its duration. Effects of short-term or chronic changes in incubation temperature on embryonic as well as on posthatching development and performance may be very different. Short-term changes in incubation temperature adapt the embryo to environmental fluctuations and, finally, improve robustness. Chronic changes leads to adaptation to the respective ambient temperature, *e.g.* cold or warm adaptation.

Chronic increase in incubation temperature during final incubation by 1°C, for instance, increased embryonic temperature as well as heat production, which is a prerequisite for an elevated thermoregulatory set point. Post-hatching, chronic warm incubated birds preferred higher ambient temperatures compared with the normal incubated control. Chronic cold incubation induced

opposite effects. Related to the prenatal temperature experiences the neuronal thermosensitivity and c-fos expression of the hypothalamus showed long-lasting alterations.

Short-term increases in incubation temperature by 1°C during final incubation decreased heat production in chicken embryos and, finally, the embryonic body temperature. Obviously, such short-term temperature stimulation has long-lasting "training effects", which improves robustness and thereby health, welfare and performance in poultry.

In broiler chicks, for instance, chronic warm incubation during the last 4 days of incubation did not affect hatching results and later performance. But, short-term temperature stimulation improved hatching rate, induced a significantly higher percentage of hatched male chicks, increased body weight at slaughter and improved feed conversion. Finally, an incubation method that includes temperature stimulation is closer to a natural correspondence with the physiological needs of the embryo, and may therefore also have positive implications in the context of animal welfare and protection.

To further develop the protocols for temperature stimulation in practice and to investigate physiological mechanisms related to improved robustness and performance, a collaborative research project on "Circadian Incubation" was started together with PasReform Hatchery Technologies (NL), in broiler chicken. In the course of this project, research will be done on central regulation of metabolism, feed intake and body weight in hypothalamic brain slices. First results of an immunohistochemical study showed long-lasting changes in the expression of Neuropeptide Y (NPY), which is involved this regulatory processes. The lower NPY expression in prenatally temperature stimulated male broiler chicks could be related to the better feed conversion and a lower basic metabolic rate compared with the normal incubated control.

Nutritional limitations during incubation period

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Knowledge about the nutritional status of the embryo during incubation is essential for understanding events that influence embryonic development, hatchability, hatchlings quality and chicken performance. While extensive knowledge exists for poultry nutrition during the growing period, the existing information on the nutrition of the broiler embryo during the incubation period is almost a ‚black box'.

Poultry embryos and hatchlings rely upon the fat, protein, carbohydrates, water, minerals and vitamins deposits in the egg compartments (albumen, yolk and shell). The ratios between the yolk and albumen compartments, their integrative relationship during incubation, their nutrients composition and the rate of their consumption, have a major part in the optimal development of the embryo.

Results show that there is a differential uptake of fat, protein and carbohydrates from the yolk during incubation period. By E17 almost 50% of the protein is absorbed by the embryo from the yolk. 65% of fat is absorbed, in a linear manner, between E11 and E17. Then, between E17 and E20, only a small amount of fat is consumed while at day of hatch, 15% of yolk fat is consumed vigorously during just 24h. Interestingly, although the level of carbohydrates in the yolk of fertile egg is minor their levels increased significantly in the yolk during incubation reaching a peak at E19. Recent results show that the yolk sac serves as the main organ that synthesizes glucose and stores it in the form of glycogen, with a quantity 20 times greater than the liver on 19E. Towards hatch, the yolk has the potential to transfer 10 times more glycogen-derived glucose to the embryo as compared to the liver. As such, the yolk may play a major role in the synthesis and storage of glucose, and its supply to the chick embryo towards hatch.

Analysing the mineral levels in the egg resources shows that Zn, Cu, Mn and P were highly consumed from the yolk (which is the major mineral reservoir) whereas from E19 the embryo has marginal mineral reserves in the yolk.

Digestion and absorption of nutrients during incubation take place by two routes .The first is absorption through the yolk sac membrane (YSM) directly into the blood. Absorption of yolk's 25–30 nm sized particles, consisting of mainly very low density lipoproteins, taken up by YSM epithelial cells by endocytosis was documented previously. Our current findings show that the uptake of yolk-nutrients, including di-and-tri peptides, amino-acids and minerals, is mediated by a variety of membrane-bound transporter proteins. Moreover, the YSM also exhibit digestion abilities similar to the digestion abilities of the small intestine. Expression profiles of nutrient transporters and digestive enzymes during incubation in the YSM and

embryonic intestine provide a basis for future research on the YSM ability and the capacity for digestion and transport of nutrients during incubation.

The second route of consuming yolk nutrients during incubation is by their transfer directly from the yolk to the intestine. Our findings show that from E17 a significant portion of the yolk enters the small intestine thorough the yolk stalk, contrary to previous research that showed this transfer only occurred posthatch. By this route the glycogen, fat, protein, minerals and vitamins are subjected to pancreatic enzyme activity and can be absorb by the embryonic intestine and support the developing embryo.

It is suggested that incubation conditions and age of the breeding flock affected the differential uptake of yolk nutrients and YSM functionality

Effect of relative humidity during incubation on embryonic mortality, chick quality, and postnatal development

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Hatching success in broiler chicken eggs is influenced by the amount of water loss from the egg during incubation (Davis *et al.*, 1988; Buhr, 1995). In previous studies, water loss was often manipulated by changing the relative humidity (RH) inside the incubator while the machine temperature was maintained at a fixed setpoint. However, it is possible that the eggshell temperatures in these studies varied between RH treatments because the heat transfer capacity of air is different at a high or low RH. Eggshell temperatures were not monitored nor controlled. Changes in embryonic mortality found in previous studies to RH or water loss during incubation may have been a reflection of changes in eggshell temperature, rather than a result of the RH or water loss itself. By incubating eggs at the optimal eggshell temperature of 37.8ºC but with variable RH, effects on embryonic mortality, chick quality and development can be attributed to RH and water loss. Therefore, this study was performed to determine embryonic mortality, chick quality and postnatal development of chickens from eggs incubated at a high or low RH while maintaining the eggshell temperature at 37.8ºC throughout incubation.

Eggs were incubated at a low (30–35%) or high (55–60%) RH from day 2 of incubation (E2) until the day of hatch (E21). Eggshell temperature was maintained at 37.8ºC throughout incubation. At E0 and E18, eggs were weighed to determine egg weight loss. The number of hatched chickens was monitored every 6 hours from 468 till 510 hours of incubation. Unhatched eggs were opened at E21 to determine the stage of embryonic mortality. Embryo or chicken weight and several parameters of chick quality and development (chick length, navel quality, organ weight and dry matter of the yolk free body mass and residual yolk) were determined at E18, E21 and 4 days posthatch.

Results showed that egg weight loss from day 0 to day 18 of incubation increased (12.7% for low RH and 9.7% for high RH; $P<0.001$), third week embryonic mortality increased (+1.6%; $P=0.02$), and hatch of fertile decreased (–2.9%; $P=0.004$) for low RH eggs compared to high RH eggs. Hatch times, variation in hatch times, and chicken weight, quality and development were comparable between RH treatments (all $P>0.17$).

Results are in accordance with the literature, which found that chickens from eggs with more than 20% weight loss at E20 will either die during incubation or hatch a chicken of the same quality and development as chickens from eggs with 14.4% weight loss (Davis *et al.*, 1988). It appears that embryos are unable to respond to the level of water loss by altering their growth rate or development. According to the literature, the only strategy an embryo has to cope with increased water loss during incubation is by maintaining its water content through selective reabsorption of water from the allantois (Davis *et al.*, 1988; Buhr, 1995). This is further evidenced by findings in the current study that egg weight loss was increased for low RH eggs compared to high RH eggs while chicken weight at E21 was comparable between treatments. This suggests that water was lost from the extraembryonic fluids rather than from the embryo and yolk. It can be concluded that incubating eggs at a high or low RH, while maintaining the eggshell temperature at 37.8ºC throughout incubation, results in increased third week embryonic mortality for low RH eggs compared to high RH eggs and comparable chicken weight, quality and development at E18, E21 and 4 days posthatch for both RH treatments.

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Clutch size variation, reproductive investment and resource allocation in the European pond turtle, *Emys orbicularis*.

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The European pond turtle is a widespread freshwater turtle of the western central Palearctic area, from Northern Europe (*i.e.*: Germany, Denmark) to south Mediterranean (*i.e.*: Southern Italy, Greece and Northern Africa). Oviparous, it lays from one clutch each year or biennially (northern Europe) to one to two clutches a year in Southern Mediterranean areas.

It is uncertain if populations could have additional reproductions. Analysing about 200 X-rays and ultrasonographic slides collected over several years and throughout the reproductive season, it emerged that clutch size (X-ray) is 6.1 ± 1.1 eggs ($n = 67$), developing follicular eggs are 9.1 ± 11.4 ($n = 190$), with average size 11.8 ± 5.9 mm. Follicular eggs (large, not shelled) are 31.6 ± 2.3 mm ($n = 65$). Clutch laying distribution ranges from 27% in May, to 67.6% in June and 5.4% in July; large follicular eggs distributions is 9.5% in May ($n = 18$), 33.7% in June ($n = 64$), 40.5% in July ($n = 77$) and 16.3% in August ($n = 31$). Differently from previous months, in August the follicula size decreased.

Investment in reproduction is basically divided in two parts, (i) producing eggs and (ii) storing additional reproductive materials for subsequent reproductive bouts. Despite a third reproduction not being recorded in Italy, the large amount of follicular in late July and August is in agreement with the possibility of a third reproductive output, may be prevented by negative climatic constraints (*i.e.* aridity).