Report for **Short Term**

Scientific Mission

CA15224

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Home Institution: University of Bern
Host/Host Institution: University of Wageningen
Start and End of STSM: 21.1.2017-21.2.2017

Investigating the effects of light during incubation on keel bone development in laying hens

1. Aims and objectives of the STSM

My short term scientific mission at the Wageningen University was carried out from January 21 until February 21, 2016, and was funded by the COST Action CA15224 KeelBoneDamage. The COST action focuses on research and activities towards the overall goal of understanding the causes of keel bone damage in laying hens and providing information to reduce its occurrence.

The aim of this short term scientific mission was to initiate collaboration between my home institution at the University of Bern, Veterinary Public Health Institute, Department of Animal Welfare, and the host institution at Wageningen University, Department of Behavioural Ecology. In my further academic career I want to focus on effects of early life experience on development and behaviour of chickens and this short term scientific mission helped me in this transition from the PhD to applying for the first post-doctoral project. I found this mission to be a great opportunity to get in touch with the experts in the chicken behaviour, associate professor dr. Bas Rodenburg, early-life development, associate professor dr. Henry van den Brandt and flight tracking and 3-D space use, assistant professor dr. Florian Muijres.

The purpose of this scientific mission was to analyse some of the data that was collected by a master student Sharon van Schaijk for her Master thesis (van Schaijk, 2017). Van Schaijk (2017) analysed the data with independent T-tests which showed the differences in development between the light and dark treatment at particular embryonic ages, though it did not show an overall treatment effect on the embryo development. I performed additional analysis of the existing data and used the results to develop a funding proposal on optimizing incubation and early life conditions to reduce the risk of keel bone fractures.

2. Description of work undertaken

Keel bone damage is currently one of the greatest welfare concerns of the laying hen industry (FAWC, 2013, 2010), which affects between 20 and 96% of all laying hens depending on the housing conditions (Wilkins et al., 2011). It is known that factors like navigational skills, physical ability and fearfulness play an important role in the development of keel bone damage (Harlander-Matauschek et al., 2015). However, the underlying mechanisms of keel bone damage are not yet fully understood. For instance, it is speculated that the environment during incubation and early-life might play a role in reducing the prevalence of keel bone damage. There are indications that providing light during incubation has a positive effect on embryonic development that extends in the first weeks after hatching (Daisley et al., 2009; Patzke et al., 2009). It has also been suggested that light during incubation improves bone mineral density (i.e. bone strength) and that

chickens exposed to light during incubation are likely less fearful (Rogers, 2010). After hatching, the complexity of the early-life environment likely also plays a role in the development of chickens (Patzke et al., 2009) and influences fearful behaviour (Jones, 1982; Rodenburg et al., 2009). However, it has been shown that chickens do not use the vertical space in complex enclosures in the first weeks of life (Kozak et al., 2016) which is likely due to unsuitability of the design, i.e. too high perches or too steep angles (EFSA Panel on Animal Health and Animal Welfare, 2015; LeBlanc and Harlander, 2016).

During my visit to Wageningen University I analysed part of the data of a preliminary study (van Schaijk, 2017): embryo size, embryo movements, melatonin levels, overall keel bone length from birds from two light treatments were available and developed hypotheses for a funding proposal to further investigate the influence of early life experience on the development of the musculoskeletal system and behaviour of chickens with the ultimate goal of reducing keel bone fractures in adult hens.

The detailed description of the methods used in the preliminary study can be found in Van Schaijk (2017); here I focus on data collection of the variables of interest: embryo length, embryo movement, melatonin level and keel bone length.

1.1 Incubation

A total number of 9600 NOVOgen white eggs were incubated at a commercial hatchery (Verbeek, Zeewolde, The Netherlands). Pigmentation of the eggshell filters light and can thus affect the influence of light (Romanoff and Romanoff, 1949; Shafey, 2004). Therefore, white eggs with low egg shell pigmentation were chosen for this experiment.

The experiment was performed in two incubators (EMKA Teggnologic 27). Temperature and humidity were monitored by measuring the eggshell temperature with data loggers placed randomly on eggs within the incubators. Conditions in the incubators (temperature, humidity, and concentration of CO_2) were kept constant for both of the provided treatments. At the end of incubation at embryonic day eighteen (E18) the eggs were candled manually to see whether the eggs are fertilized. After candling, remaining eggs were brought to the hatch room for hatching.

1.2 Treatments

Each incubator provided different light schedules exposing eggs either to constant darkness (24D) or constant light (24L). The eggs (4800 per treatment) were randomly assigned in of one of the two incubators with the specific treatment. Each incubator was equipped with six trays of 800 eggs each. Treatments started on embryonic day zero (E0) and were provided continuously during incubation, and afterwards in the hatch room. The eggs were candled on E18 before the transfer to the hatch room. For the light treatment, approximately 1500 lx light was provided by strips of cool-white light emitting (LED) lights placed at 10 cm above the eggs.

1.3 Data collections

Measurements were taken on embryonic days 10, 12, 14, 16, 18, and 21 (at hatch) by the same person. All subjects used for this study (n=240) were removed randomly from the incubator and were labelled before the data collection. During all data collections, the lighting conditions in the data collection room were adjusted to the treatment, i.e. measurements of 24D were carried out in darkness and for 24L eggs the room was lit with

the white light. Exact embryonic days for specific data collections are presented in Figure 1. Data collections included measurements of:

1. general development (body weight, chick body length, beak length, third toe length, navel condition and viability test; 10 eggs/treatment/embryonic day=100 samples).
2. embryo motility (heart rate and embryo movements; 10 eggs/ treatment/embryonic day=60 samples). To be able to see the embryos, the eggs were windowed, enhancing contrast in the image. Locomotor activity was recorded for two minutes.
3. melatonin concentration (10 eggs/treatment at six different time points during 24 hours=120 samples).
4. keel bone development (size, ossification; 12 eggs/treatment/embryonic day=120 samples).

Some data was collected from the eggs/embryos, e.g. general development and embryo motility on E10-E18.

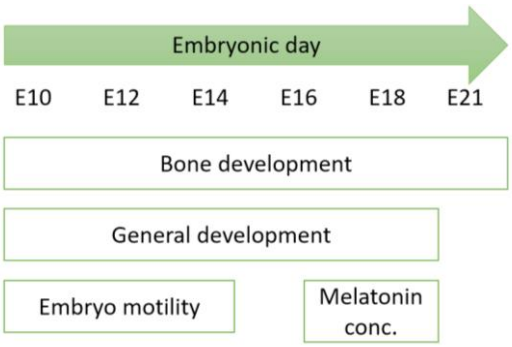

Figure 1: data on general development, bone development, embryo motility and melatonin concentration was collected from E10 to E21.

1.3.1 Embryo length

Embryo length of total n=100 embryos was measured on E10, E12, E14, E16, and E18. Embryo length was defined as the length from the tip of the beak to the implantation of the nail on the third (Figure 2).

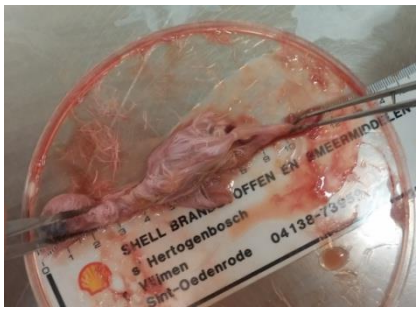

Figure 2: Embryo length was measured from the tip of the beak to the implantation of the nail on the third toe.

1.3.2 Embryo movements

Embryo movement is likely associated with bone development (van der Pol et al., 2015). Hence, the frequency of embryo movements was measured on E10, E12, and E14 in the same eggs as used for the embryo length measurements (n=60). At the embryonic age chosen the frequency of motor activity of the embryos is likely the highest (Wu et al., 2001). To measure embryo movements, the eggs were illuminated from beneath with an

infrared light, creating a silhouette of the embryo. Locomotor activity was recorded for two minutes with a high-speed camera (Photron APX; 50 fr/sec. 1024X1024 pxl). The eggs were windowed to enhance contrast in the image. Video observations were used to assess embryo movements.

1.3.3 Melatonin concentration

Melatonin has been shown to be involved in the bone formation and remodelling (Liu et al., 2013) Melatonin concentration was assessed only on embryonic day 18 and at six points during a 24-hour-period. For identifying melatonin concentration, in total 120 pineal glands were collected (ten glands per treatment per time point). The dissected pineal glands were frozen in liquid nitrogen, stored on dry ice of -80 ^{0}C, and shipped to dr. M. Zeman in Slovakia, who measured melatonin levels in the pineal glands.

1.3.4 Keel bone length

Bone development of in total 120 embryos was measured on E10, E12, E14, E16, E18 and at hatch. For E10, E12 and E14 the same ten eggs as in the embryo length and embryo movements were used. For E16 and E18 the same eggs as in the embryo length were used. At the hatchery, the embryos were removed from their egg and yolk sack and the viscera were removed. The embryo samples were fixated in formaldehyde and transported to the laboratory where the head, neck, legs, lower part of the torso, and the ribs were removed.

A staining protocol was developed and is described in van Schaijk (2017): Appendix 7.3.4. After staining the keel bones, the following variables were measured with a digital calliper: lengths, widths and heights of the keel bone and its components. More detailed information about the variables can be found in van Schaijk (2017): Appendix 7.4.3.; here I focus only on keel bone length.

1.4 Statistics

For statistical analysis, R studio was used (RStudio, 2012). Predictors were treatment (light schedule), embryonic age, and the interaction of these terms. For statistical analysis of melatonin level, treatment and time of day were independent variables and tray an experimental unit. If not stated otherwise, data was analysed with a generalized linear mixed model assuming a Gaussian distribution. Embryo movements had a binomial distribution (move/still). Stepwise backward elimination based on Akaike's information criterion (AIC) was used to determine the best fitting model (Bolker et al., 2009) and model terms were considered significant at $p \leq 0.05$. The fit of each model was examined using Q-Q plots of the residuals, and plots of predicted versus fitted values.

3. Main results

1.5 Embryo size

The length of embryos was not affected by the treatment ($p=0.432$), and the embryos grew with embryonic age (Figure 3; $T=40.04$, $p<0.001$).

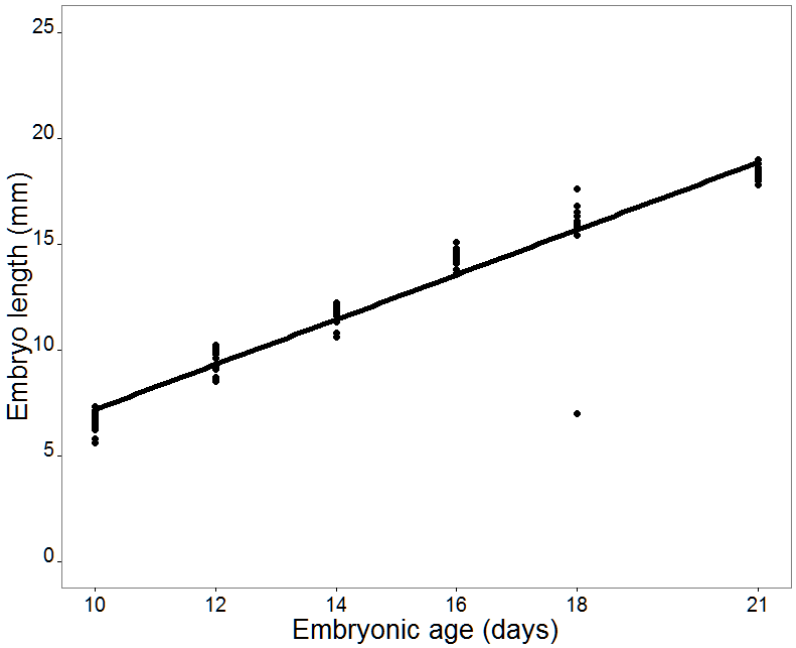

Figure 3: Embryonic age effect on the length of embryos.

1.6 Embryo movements

The number of embryo movements did not relate to the treatment (p=0.105) or embryonic age (p=0.406). In total, 24L embryos moved eight and 24D three times in 30 measurements per treatment.

1.7 Melatonin level

24D embryos had lower overall melatonin concentration than 24L embryos (Figure 4; T=-2.72,p=0.008). There was a tendency for an increase in melatonin concentration during the day (p=0.062).

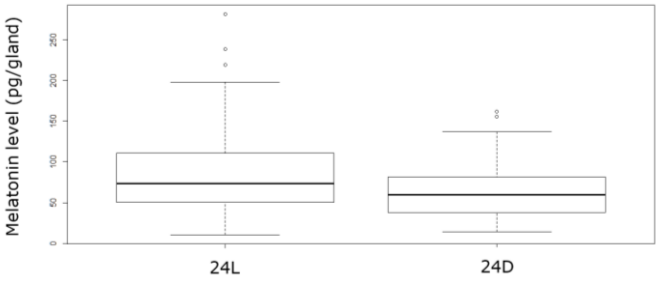

Figure 4: melatonin concentration

1.8 Keel bone

24D treatment resulted in overall shorter keel bones (Figure 5; Z=-2.73, p=0.007) and in both treatments, keel bones got longer with embryonic age (Z=51.61, p<0.001).

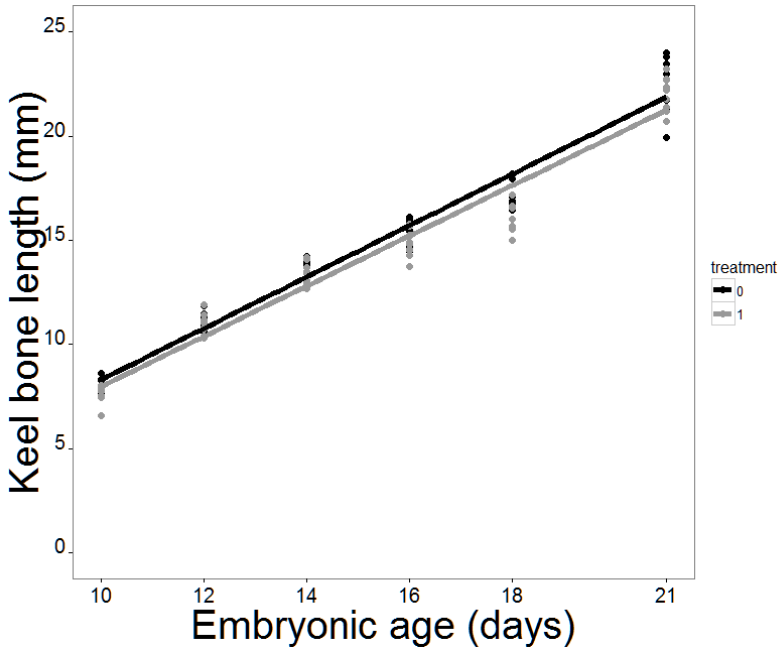

Figure 5: The effect of light on keel bone length. Eggs incubated with daily 24-hour light exposure (black) vs. 24-hour darkness (grey).

We found the results of melatonin level and keel bone length especially interesting. Melatonin level in adult hens has a diurnal rhythm with its peak in production in the dark period. While the lack of diurnal rhythm is less surprising since the eggs had a constant exposure to the treatment, it would be expected that embryos incubated in the darkness (24D) would have higher melatonin levels than 24L embryos. We assume that exposure to light is important for the development of the pineal gland. Since melatonin is an important hormone in bone formation and remodelling (Liu et al., 2013), we predict that higher melatonin excretion in 24L birds could result in a lower prevalence of keel bone fractures and/or faster healing of keel bone damage in adult hens.

Further, 24L embryos had larger keel bones, however, this might have double implications. Larger bones indicate faster development of the musculoskeletal system; though faster development does not necessarily lead to less keel bone damage. Young soft not yet fully calcified bones are less prone to fractures and it is the ossification of the keel bone that may play a greater role in reducing the prevalence of keel bone fractures than the size of the bones. It is thus important to test whether faster development of the musculoskeletal systems affects the prevalence of keel bone fractures in adult hens.

4. Outputs produced (e.g. academic paper, funding application, new dataset etc.) and future plans, including potential future publications

Together with Dr. Bas Rodenburg, Dr. Henry van der Brandt and Dr. Florian Muijres, I used the preliminary findings described above to develop an application for funding that was submitted to the Wageningen Institute of Animal Sciences (3-year post doc proposal). The project aims to investigate the effects of light during incubation and housing conditions during early life on the prevalence of keel bone fractures in adult laying hens, by studying

the effects of incubation and early-life conditions on physical and behavioural development.

5. Future collaboration possibilities with the host institution

Both, the host and home institutions, are leading institutions in laying hen welfare, they both focus on finding causes and solutions for keel bone fractures in laying hens and they both have their own expertise which offer plenty of possibilities for collaboration. For instance, while the home institution focuses in tracking hens directly in commercial systems, the host institution has a possibility to use high speed cameras and equipment for accurate sensor-based recording of location, activity and proximity in laying hens in a more controlled laboratory environment. Through the COST Action KeelBoneDamage, we will explore further opportunities for collaboration between Bern and Wageningen University. Furthermore, Janja Sirovnik is applying for a post doc position at Wageningen University.

6. Literature

Bolker, B.M., Brooks, M.E., Clark, C.J., Geange, S.W., Poulsen, J.R., Stevens, M.H.H., White, J.-S.S., 2009. Generalized linear mixed models: a practical guide for ecology and evolution. Trends Ecol. Evol. 24, 127–35. doi:10.1016/j.tree.2008.10.008

Daisley, J.N., Mascalzoni, E., Rosa-Salva, O., Rugani, R., Regolin, L., 2009. Lateralization of social cognition in the domestic chicken (Gallus gallus). Philos. Trans. R. Soc. B 364, 965–981.

EFSA Panel on Animal Health and Animal Welfare, 2015. Scientific Opinion on welfare aspects of the use of perches for laying hens. EFSA J. 13, 4131–4170. doi:10.2903/j.efsa.2015.4131

FAWC, 2013. An open letter to great britain goverments: Keel bone fracture in laying hens [WWW Document].

FAWC, 2010. Opinion on Osteoporosis and Bone Fractures in Laying hens [WWW Document]. London Farm Anim. Welf. Counc.

Harlander-Matauschek, A., Rodenburg, T.B., Sandilands, V., Tobalske, B.W., Toscano, M.J., 2015. Causes of keel bone damage and their solutions in laying hens. Worlds. Poult. Sci. J. 71, 461–472.

Jones, R.B., 1982. Effects of early environmental enrichment upon open-field behaviour and timidity in the domestic chick. Behav. Processes 5, 161–172.

Kozak, M., Tobalske, B., Martins, C., Bowley, S., Wuerbel, H., Harlander-Matauschek, A., 2016. Use of space by domestic chicks housed in complex aviaries. Appl. Anim. Behav. Sci. 181, 115–121.

LeBlanc, C.N., Harlander, A., 2016. Development of Domestic Fowl Locomotion over Inclined Surfaces and Use of Anticipation Strategies.

Liu, J., Huang, F., Hong-Wen, H., 2013. Melatonin Effects on Hard Tissues: Bone and Tooth. Int. J. Mol. Sci. 14, 10063–10074.

Patzke, N., Ocklenburg, S., van der Staay, F.J., Guüntürkün, O., Manns, M., 2009. Consequences of different housing conditions on brain morphology in laying hens. J. Chem. Neuroanat. 37, 141–148.

Rodenburg, T., Uitdehaag, K., Ellen, E., Komen, J., 2009. The effects of selection on low mortality and brooding by a mother hen on open-field response, feather pecking and cannibalism in laying hens. Anim. Welf. 18, 427–432.

Rogers, L.J., 2010. Relevance of brain and behavioural lateralization to animal welfare. Appl. Anim. Behav. Sci. 127, 1–11.
RStudio, 2012. RStudio.
van der Pol, C., Molenaar, W.R., Buitink, C.J., van Roovert-Reijrink, I.A.M., Maatjens, C.M., van den Brand, H., Kemp, B., 2015. Lighting schedule and dimming period in early life: consequences for broiler chicken leg bone development. Poult. Sci. 94, 2980–2988.
van Schaijk, S., 2017. Early effects of lighted incubation on embryonic keel bone development in layer breeder eggs.
Wilkins, L.J., McKinstry, J.L., Avery, N.C., Knowles, T.G., Brown, S.N., Tarlton, J., Nicol, C.J., 2011. Influence of housing system and design on bone strength and keel bone fractures in laying hens. Vet. Rec. 169, 414. doi:10.1136/vr.d4831
Wu, K.C., Streicher, J., Lee, M., Hall, B., Müller, G., 2001. Role of motility in embryonic development I: Embryo movements and amnion contractions in the chick and the influence of illumination. J. Exp. Zool. 291, 186–194.