Maternal care and selection for low mortality influence mineralocorticoid receptor levels and behavior in laying hens

Jasper Heerkens

March 2010

University Utrecht, Faculty of Veterinary Medicine

Department of Farm Animal Health, Program Emotion & Cognition

and

Adaptation Physiology Group, Wageningen UR

Maternal care and selection for low mortality influence mineralocorticoid receptor levels and behavior in laying hens

Jasper Lidwina Thomas Heerkens
Reg.nr.: 810828316090

ADP-80436, 36 ECTS

Supervisor WUR: Dr. Bonne Beerda
Supervisor UU: Dr. Rebecca Nordquist

March 2010

Adaptation Physiology Group, Wageningen University
and
University Utrecht, Faculty of Veterinary Medicine
Department of Farm Animal Health, Program Emotion & Cognition

Abstract

Feather pecking and cannibalism are major welfare problems in commercial laying hen husbandry. Breed differences in feather pecking indicate a genetic component. Further, early-life conditions play an important role in the development of these behaviors. A selection experiment was initiated selecting on low mortality in group housing for four generations. This resulted in a low mortality line (LML) and an unselected control line (CTL). Previous studies have shown that chickens from the low mortality line show a number of behavioral and physiological differences compared with their control counterparts, including altered whole-blood serotonin levels, plasma corticosterone levels and open field behavior. Here, behavioral differences between selection lines were investigated further and it was tested whether interactions between genetic selection and maternal care determined on mineralocorticoid receptor (MR) levels, which could have implications for sociality, fearfulness and thus welfare.

Brains from the second generation of LML and CTL, reared either with or without maternal care, were collected at 40 weeks of age and immunocytochemically stained for MR. No differences were seen between selection lines in MR receptor expression. Brooding hen presence lead to MR lateralization in the nidopallium caudolaterale + posteriosis amydalopallii, and tended to increased MR levels in the left hemisphere in the nidopallium caudolaterale + posteriosis amydalopallii. No differences were seen in the hippocampus. At 1-4 weeks of age, behavior of fourth generation LML birds and CTL birds was studied in an open-field test, T-maze test, holeboard test and in a voluntary approach test. No line differences in sociality and fearfulness were found in the open-field test. The holeboard tests revealed no line differences in working memory and reference memory. T-maze results showed increased sociality at 12-16 days of age: the LML spent more time with conspecifics (LML:181±38s; CTL:0±0s) in the T-maze and showed a learning curve in time needed to find conspecifics in the T-maze ($p<0.001$ for cubic trend), whereas the control line did not show any learning. At 26 days the LML were less fearful as they had a shorter latency to approach a familiar human in the voluntary approach test [LML: (mean ± standard error of the mean) 17±6s CTR: 84±14s]. Thus, brooding hen presence during the rearing period leads to increased MR expression in the left hemisphere and increased MR receptor lateralization in later life. Selection for low cannibalism in laying hens leads to increased sociality and reduced fearfulness at young age.

Introduction

Feather Pecking and Group Selection

In commercial systems large groups of laying hens (*Gallus gallus domesticus)* are reared and kept in unnatural housing conditions, subjecting the animals to numerous environmental stressors, thereby challenging the adaptive capacity of the animals. Laying hens that are not able to cope with the environmental stressors often develop maladaptive behaviors, which are manifestations of impaired animal welfare. Feather pecking and cannibalism are maladaptive behaviors that develop from 18 weeks onwards and may be redirected ground pecking behavior (Blokhuis 1986) or pecking during dustbathing (Vestergaard and Lisborg 1993) and are considered as a major welfare problem in laying hens (Rodenburg et al. 2009; Rodenburg et al. 2008). Also, feather pecking may be related to "social exploration". The incidence of feather pecking increases when chicks are presented with unfamiliar chicks and the direction of pecks are against these unfamiliar individuals (Riedstra & Groothuis, 2002). Rodenburg et al. (2008) reviewed the literature about feather pecking and cannibalism and stated that three important factors (genetic background, early-life history, and environmental factors) must be taken into account to approach the problem of feather pecking and cannibalism.

Considering the genetic background, Ellen et al. (2007) proposed a group selection method in which selection of individually housed laying hens was based on group performance of relatives in family groups. The outcome of selection has a consequence that can be split in two components (Griffing et al. 1967). The first component embodies the phenotype of the individual as direct effect of its genotype. The second component is the effect of the individual genotype on the phenotype of other individuals. The genotypic effect of an individual on the phenotype of others is referred as associative effect of that particular genotype. To genetically select on traits, like propensity to develop feather pecking and cannibalistic behavior, a selection method is required that has both a beneficial direct effect on the genotype as well as a desirable associative effect.

Feather Pecking Associated Behaviors

Fearfulness and social motivation, both tested in the open-field test, are traits which are associated with the propensity to develop severe feather pecking and cannibalistic behavior in chickens (Jones et al. 1995; Rodenburg et al. 2004). Behaviors shown during the open-field test are a compromise in opposing tendencies of minimizing detection by predators and of returning to flock-mates. The first tendency reflects fearfulness, the latter tendency reflects social motivation or sociality (Rodenburg et al. 2004). Silence and freezing of chicken in the open-field test has been linked to fear. Young chicks that are more inactive in the open-field test at young age are more active in the open-field test at adult age and develop more feather pecking behavior and cannibalism (Rodenburg et al. 2009; Rodenburg et al. 2004).

Jones et al. (1995) states that vocalizations and distress calls must be regarded as socially-motivated behavior patterns. More pronounced social motivation may increase the likelihood of the isolated chick to reinstate social contact. Jones et al. (1995) found that young chicks of a low feather pecking line (LFP) showed shorter latency to walk, vocalized sooner and showed less freezing behavior in the open-field test compared to their high feather pecking line (HFP) counterparts. Rodenburg et al. (in press 2009) also found that chicks from a low mortality line (LML) expressed more distress calls. These differences found in open-field tests indicate that propensity of developing feather pecking is linked to in differences in social motivation and fearfulness of chickens.

Feather Pecking and Early-life History

Besides the genetic background, early-life history and environmental factors play a role in the development of feather pecking (Rodenburg et al. 2008). Maternal care during the rearing period is thought to be an important early-life factor, as the brooding hen teaches to peck at more rewarding substrates (Perré et al. 2002). Rodenburg et al. (in press 2009) tested the effects of selection on low mortality in combination with brooding by a mother hen on open-field behavior, feather pecking and cannibalism in chickens. They found that LML chickens which were reared with a brooding hen showed a shorter latency to stand up and to walk and were more active in the open-field. This observation suggests that both selection on low mortality and brooding hen presence contribute to less fearful chickens with stronger explorative motivation.

Early-life History and Neuroanatomy

Housing conditions affect the morphology, chemistry and physiology of the central nervous system and the physical abilities of animals (Diamond 2001; van Praag et al. 2000). The behavior, physiology, and brain morphology of laying hens may be sensitive to the modulatory effects of these rearing and housing conditions. The famous research of Harlow and Harlow (1966) described the deleterious effects of social deprivation as a specific kind of impoverished environment in socially living animals, and showed severe impairments in social and sexual behavior of rhesus monkeys raised in social isolation. Social deprivation also had structural neurochemical effects in forebrain areas of young chickens (Gruss and Braun 1997). In modern poultry husbandry, the young chicks never come in contact with their parents or other elder conspecifics. This deters the young animal from observational and social learning of normal behavior, such as efficient foraging by pecking at profitable food items and redirecting attention away from harmful or non-profitable items (Nicol 2006).

Environmental enrichment affects the hippocampus, whereas the mammalian prefrontal cortex (PFC) especially reacts to social deprivation. Chronic stress causes morphological and functional changes in both areas. Stress suppresses neurogenesis in the hippocampus and is accompanied by modified learning and memory abilities (Mirescu and Gould 2006). Chronic stress also induces atrophy in the PFC and impairs both working memory and behavioral flexibility (Cerqueira et al. 2001).

The Mineralocorticoid Receptor

Joëls et al (2007) extensively describes the role of the mineralocorticoid receptor (MR) in relation to corticosteroids and stress responses. The MR and the glucocorticoid receptor (GR) are abundantly expressed in the hippocampus and the nidopallium caudolaterale (NCL), a functional analogue of the mammalian prefrontal cortex. These receptors are activated by the binding of corticosteroids which are secreted from the adrenal glands. The natural ligand corticosterone has a much higher affinity for the MR than for the GR. The high affinity of the MR for corticosterone ensures high occupancy and activation of MR during low circulating hormone levels. Hippocampal MR output on inhibitory neurons of the hypothalamus leads to stronger inhibition of CRH-producing cells in the paraventricular nucleus (PVN). MR prominently contributes to mechanisms that drive the initial phase on the onset of the stress reaction by excitatory output from the hippocampus on inhibitory interneurons of the hypothalamus and therefore enhances

inhibitory input to the CRH-producing cells in the PVN. Maze tests have shown involvement of hippocampal MR in cognitive processes, such as assessing novel situations and novel object reactivity, behavioral flexibility and selection of appropriate behavioral responses to deal with a challenge (Joëls et al. 2007).

Lateralization

The brain is a highly plastic organ which maintains its plasticity throughout the entire lifespan of an animal (Frick and Fernandez 2003) and this plasticity may contribute to lateralization of the brain. Lateralization is thought to increase brain efficacy and promote survival under natural conditions. It can be seen as an adaptation and specialization of certain brain areas, allowing the animal to perform two different tasks simultaneously (Rogers et al. 2004). Daisley et al. (2009) reviewed that lateralization improves social (re)cognition in chicks. Patzke et al. (2009) showed that in adult laying hens housing conditions (battery cages, small littered ground pen, and free range system) influenced brain morphology, although the differences were minimal.

The hippocampal substructures and nidopallium caudolaterale (NCL), a functional analogue of the mammalian prefrontal cortex, undergo changes during chronic stress and social stress. Patzke suggests that the pre-pubertal rearing conditions are of great importance in lateralization. Lateralization of the brain is suggested to be a possible beneficial outcomes of group selection (LML vs. CTL) and different rearing conditions (brooding vs. non-brooding).

Aim of the Study

Animals that are calm, not fearful and social are preferred in large commercial systems, as they show less feather pecking and cannibalism. Therefore, the ultimate purpose of the greater project, including this thesis, is to improve breeding programs and rearing conditions for laying hens. This will lead to better adaptation of the animals to its housing conditions and living in large groups in commercial systems, thereby increasing its welfare. Besides preferred behavioral traits the degree of lateralization, as an index for brain development, could mirror the degree of welfare of a chicken (Manteca 1998).

The aim of this study was to analyze the impact of group selection (LML vs. CTL) on behavior, in particular social behavior and fearfulness. Underlying the possible behavior

differences might be lateralization of the brain. Lateralization caused by group selection or brooding hen presence during the rearing period were assessed by analyzing neurochemical morphology in the laying hen brain (resp. MR in hippocampus and NCL).

As mentioned in the introduction, open-field test results have been consistent, or at least comparable, in several behavioral studies on selection for low mortality. However, the T-maze, voluntary approach test and holeboard test have never been used in these selection studies. Therefore, these behavioral tests were also performed to establish and test suitability of these behavioral tests for learning and memory (holeboard, Arts et al. 2009), sociality (T-maze, Jones et al. 1999) and fearfulness (voluntary approach test) in chickens that have been selected for low mortality. The open-field test was also implemented in the study as previous studies (Römkens, 2009; Rodenburg et al. 2009; Rodenburg *in press* 2009) did not test in 7 day old chicks and to investigate the effects of selection over four generations.

Material and Methods

Animals

Animals for the brain morphology

Prior to the start of this project the two different selection lines were generated: the low mortality line and control line. Both lines originated from the same purebred White Leghorn layer line from ISA B.V., the layer breeding division of Hendrix Genetics. The low mortality line animals consisted of hens that were selected for two generations on low cannibalism related mortality. The animals were housed and reared either with or without a broody hen, leading to four different treatment groups (low mortality with broody hen, control line with broody hen, low mortality line without broody hen, control line without broody hen). The fostering procedure and keeping conditions were as described in Rodenburg *in press* 2009. A total of 28 brains (N=7 per treatment group) of 40 week old hens were collected and fixed in 4% paraformaldehyde. At a later stage the brains were embedded in gelatin, cut in 40µm thick slices on a vibratome and stored in tubes with 0.12M PBS and 0.1%NaAz.

Animals for the behavioral tests

For the behavioral experiments, chicks of the fourth generation of selection for low morality were used. Thirty fertilized eggs from the low mortality line and 30 fertilized eggs from the control line were obtained from ISA B.V. The eggs were incubated and hatched at the Faculty of Veterinary Medicine of Utrecht University. After hatching, the day-old chicks were moved to the Utrecht University farm animal facility 'De Tolakker'. After determining the gender, the female chicks (N=10 per selection line) were kept for the experiments, the male chicks were humanely euthanized. The chicks were kept in groups of 10 animals in pens measuring 1.12m X 1.20m X 0.70m (length x width x height). The floor of the pen was covered with wood shavings, food and water were *ad libitum* supplied, and a 400W overhead heat lamp (normal light, no infrared) provided warmth. The protocol was approved by the Utrecht University Board for studies in experimental animals, following the Dutch law on animal experiments. This complies with the ETS123 (Council of Europe 1985) and the 86/609/EEC Directive.

Immunocytochemistry mineralocorticoid receptor

Immunocytochemical detection for mineralocorticoid receptor (MR, monoclonal mouse IgG, Abcam) was performed with free-floating slices. Based on Patzke et al. (2009) the

nidopallium claudolaterale (NCL) and two different hippocampal areas, the dorsalmedial hippocampus (DM) and the medial hippocampus (mHp) were initially selected for analysis. For the brain lateralization of mineralocorticoids in the NCL, mHp and DM, slices were coded for group and hemisphere to allow blind analysis. Slices were washed three times for 5 min. with PBS before incubating with 5% normal goat serum (NGS) in 0.05 M TBS + 0.05 Tween-20 (TBS-T) at room temperature for 1 hour to block non-specific binding sites. After blocking the slices were incubated with the primary antibody solution, a mouse monoclonal MR antibody (Abcam) (MR 1/1000 in TBS-T + 1% NGS + 0.05% bovine serum albumin) overnight at 4°C. Endogenous peroxidase was deactivated with 0.3% H_2O_2 in methanol for 10 min. at room temperature. Then the slices were incubated with biotinylated goat anti-mouse IgG secondary antibody (Vector Laboratories, 1/200 in TBS-T + 1% NGS), for 1 hour at 37°C. After washing three times for 5 min. in 0.05M PBS, the slices were incubated in an avidin-biotin-peroxidase solution (Vectastain ABC-Elite kit, 1/100 in TBS) for 45 min. at room temperature. Peroxidase-activity was detected by using 3'3-diaminobenzidine (DAB, Sigma) with 0.02% H_2O_2. The slices were mounted on gelatinized slides, dehydrated in alcohol (70, 90 and 100%) and xylene (100%) and coverslipped with DePex (Serva Electrophoresis). The NCL and hippocampal areas were analyzed in 3 sections ranging from A 6.4 to A 5.8 according to the stereotaxic atlas of the chicken brain by Kuenzel and Masson (Fig.1). To determine lateralization, the left and right hemisphere were separately measured. Multiple Image Alignment (MIA) was used to measure MR intensity through mean color intensity detection using the Cell^D Software package (Olympus, Hamburg, Germany). A single MIA consisted of 25-40 tiled pictures (Olympus BX51, microscope field 10x) that provided a detailed picture of the area of interest (fig. 7).

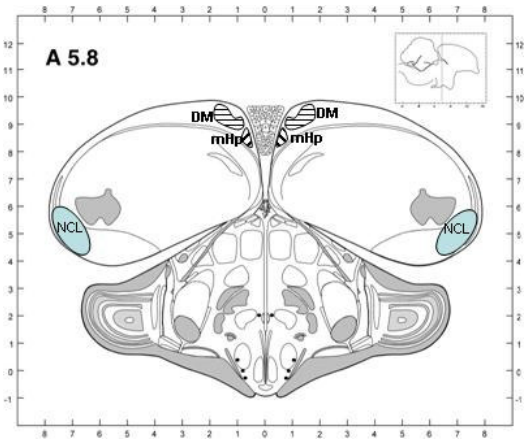

Fig. 1. Schematic view of the chicken brain section at A5.8 (Kuenzel and Masson 1988; www.avianbrain.org[1]) with the dorsalmedial hippocampus (DM), medial hippocampus (mHp) and nidopallium claudolaterale (NCL) + posteriosis amygdalopallii (PoA) .

The area referred to as NCL in figure 1 consisted of the ventral part of the NCL and the dorsal part of the nucleus posteriosis amygdalopallii (PoA). The PoA is the functional analogue of the mammalian amygdala. The PoA is a limbic area related to control of fear behavior (Saint-Dizier et al. 2009; www.avianbrain.org[2]). Therefore, the results and discussion will continue on the limbic area ranging from A5.8-6.4 containing the ventral part of the NCL and the dorsal part of the PoA, further referred to as NCL + PoA. For each area measured (NCL+PoA, mHp and DM), the effects of brooding hen and selection line on MeanL+R and $|AbsL-R|$ were assessed by analysis of variance (ANOVA) with the between subjects factors Presence of Brooding Hen (presence vs absence) and Selection Lines (LML vs CTL). The effects of these factors on lateralization of MR expression were analyzed by repeated measure ANOVA with the between subjects factors Presence of Brooding and Selection Lines and the within subjects factor hemisphere (left vs right hemisphere). .

Western Blotting Mineralocorticoid Receptor

Tissues from individual chickens [left hemisphere (HSL), right hemisphere (HSR), cerebellum (CB), remaining of brain (RB) and pig kidney lysate (KL) as positive conrtol] were pulverized in a freezing morter and diluted in RIPA buffer (RIPA, phosphatise, protein inhibitor, protease). Samples were homogenized using pottersticks and homogenization through a syringe needle (22G). Homogenates were centrifuged for 5 min at 10,000 RPM at 4°C. Protein concentrations were determined in the supernatants

using Lowry BSA protein assay kit (Bio-Rad). Homogenates were diluted with lysis buffer to a concentration of 3 µg/µl per lane for HSL, HSR, CB and RB and 1 µg/µl per lane for KL in a total volume of 20 µL. SDS page sample buffer (5x Laemmli buffer, Bio-Rad) was added to the samples. After briefly vortexing, samples were incubated for 5 min at 95°C. Precast 0.75 mm SDS-7.5% acrylamide gel (Bio-Rad) was loaded with 20 µL sample, alongside 7 µL of protein standards (BenchMark, 10-190 kDa). Samples were run at 18 mA for 1.5 h, then transferred to a 0.45 µm nitrocellulose membrane (Trans-Blot, Bio-Rad) in 1X transfer buffer (Bio-Rad) at a constant voltage (100 V) for 1 h. Nitrocellulose transfer membranes were placed in blocking solution [5% non-fat dry milk in Tris buffered saline (TBS; 1 mM Tris-HCl, 15 mM NaCl, pH 8.0)] containing 0.1% Tween-20 for 1 h at room temperature. The blots were incubated overnight at 4°C in mouse monoclonal MR antibody (Abcam) diluted 1:500 in TBS-T containing 5% non-fat dry milk (Bio-Rad). Blots were washed with TBST and then incubated with 1:5000 goat anti-mouse peroxidase secondary antibody (DAKO) in 5% non-fat dry milk in TBST for 1 hour at room temperature. Blots were washed with TBST and incubated with SuperSignal West Dura Stable Peroxidase chemiluminescence substrate (Thermo Scientific) and exposed processed under the ChemiDoc apparatus (Bio-Rad).

Open-field test

At 7 days of age, each bird was tested in an open-field test for 10 min. The open-field consisted of an observation pen measuring 1.22 x 1.22 x 0.74m (width x length x height) of Medium-density fibreboard (MDF). The floor was divided equally in 5x5 squares by white markings. The sessions were recorded with a camera that was attached to the ceiling, allowing the observer to record the behavior from a video-screen in an adjacent room (The Observer software package, Noldus Information Technology B.V., Wageningen, The Netherlands). Latency to cross the first square, total time spent walking, total number of squares crossed and total number of distress calls were recorded using focal sampling continuous recording. Testing order was randomized (SAS PLAN procedure). After being caught gently and placed in the middle of the Open-field the 10 min. observation started. After the 10 min. observation the bird was immediately returned to its home pen. All tests and observations were performed by a single person between 9.00 and 15.00h.

For each variable, the effects of selection line on latency to cross the first square, total time spent walking, total number of squares crossed and total number of distress calls

were assessed by analysis of variance (ANOVA) with the between subjects factor Selection Lines (LML vs CTL).

T-maze

The T-maze apparatus (Fig. 2), made out of MDF, consisted of a starting chamber (A in Fig. 2), a corridor (B in Fig. 2) and a perpendicular arm (C in Fig. 2). At the junction of the T corridor a mirror with a diameter of 16 cm (M in Fig. 2) was situated to facilitate movement of the chick from the starting chamber towards this point. Linked to the perpendicular arm were two compartments (D in Fig. 2) that were in open contact through an opening with wire mesh to a box (E in Fig. 2) containing two or no companions during the test. Birds were placed in the maze for 5 minutes on 3 consecutive days prior to the initial experiment to get familiar with the maze. Each bird was tested individually on 5 consecutive days between the age of 12 and 16 days. Two hens of the same line as the test bird were placed in the left or right box E (Fig. 2); the other side remained empty. For each individual bird, the side with companion birds was the same throughout all experimental days. A bird was caught gently and placed in the starting chamber. The actual test started following removal of a partition between the starting chamber and corridor. Testing order was randomized (SAS PLAN procedure). A single session lasted for 10 min. in which latency, frequency and duration of leaving and entering compartments A, B, C and D (left and/or right) were recorded. Immediately after the session birds were returned to their home pen.

Most or all animals of the CTL did not leave the starting position, revealing ceiling scores. In this case, comparison between lines was not indicated. Instead, we investigated whether the behavior of LML changed across days in this test. To this end, an analysis of variance with the within subjects factor Days (days 1-5) was performed. In addition, orthogonal trend coefficients were calculated per animal of the LML strain. These coefficients were analyzed by an ANOVA with the repeated measures factor Days (day 1 to 4, because on day 5 no conspecifics were present). In this way, the plot of behavior on successive days was broken down into a linear, quadratic and cubic component, and the presence of particular trends was detected using one-sample t-statistics (i.e. t-tests revealed whether a particular trend coefficient deviated from zero). A P-value of <0.05 was considered to represent significant differences.

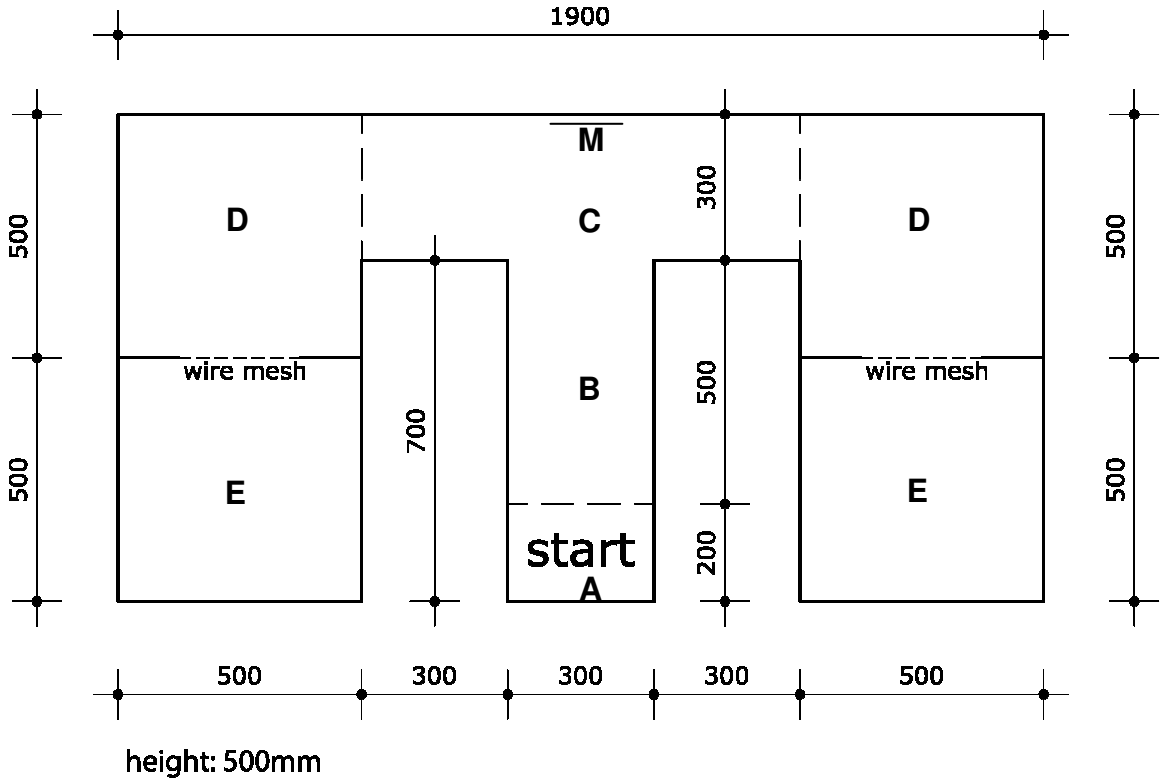

Fig. 2. T-maze outline. Starting chamber (A), corridor (B), perpendicular arm (C), compartment were companions were visible if present (D), compartments containing companions (E), mirror (M).

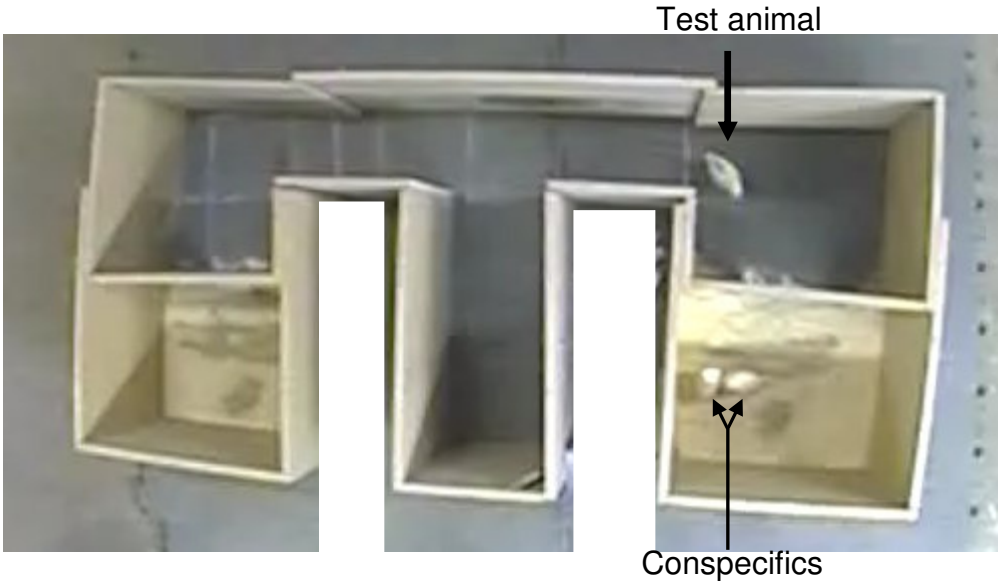

Fig. 3. Animal in T-maze experiment. Top right the test animal made contact with conspecifics (below right).

Holeboard

The holeboard testing arena measured 2.44 m x 2.44 m. On the floor were nine MDF boards, measuring 19 cm x 19 cm. In the center of each board was a vividly red colored cup, diameter 7 cm and height 5 cm. The distance between the cups was 70 cm. Around the vividly red colored cups a circle was drawn with chalk, with a diameter of 50 cm (fig. 4). The chicks were allowed to habituate to the holeboard system during 5 days of training trials. During the training trials all the cups were baited with one mealworm per cup. In the first training trial the chickens were tested in pairs of the same line, as it was assumed that social support reduces their anxiety and to stimulate copying behavior. A training trial had a time limit of 5 min, or ended when all 9 mealworms were eaten. From the age of 25 days the chickens were individually tested for 6 working days. Two hours prior to the testing, the food was removed from the home cage to stimulate the motivation to search the food rewards. The chicken was placed in the lower right corner where the starting point was marked by a chalk line (fig. 4.). In the testing trials 3 of the 9 cups were rewarding for the chickens as each of these cups contained a mealworm. The other six cups remained empty. For each chicken the same cups were baited during all trials. A single trial had a time limit of 5 min. or ended if all 3 mealworms were found (and eaten). The chicken was then removed from the testing arena for 1 minute after which a new trial started. This is expected to “reset” the short-term memory of the chicken. Latency to visit the first cup, trial duration, all cups visited and revisited, and number of worms eaten were registered. Table 1 lists all measurements taken during the trials. Testing trials were recorded with a video camera placed above the apparatus. Testing order was randomly determined using the SAS PLAN procedure. Working memory was determined by dividing the number of mealworms eaten through the total number of visits to the rewarded set of cups. Reference memory was determined by dividing the number of visits to rewarded cups through the total number of visits to all cups. An analysis of variance with the between subjects factor Line (LML vs. CTL) and the within subject (repeated measures) factor Days (days 1-6) was performed using the GLM procedure (SPSS 16.0 2007). A P-value of <0.05 was considered to represent significant differences.

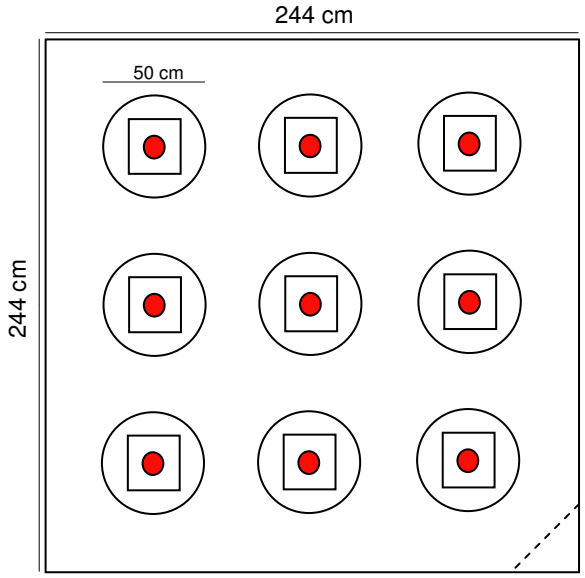

Fig. 4. Hole-board design. Per session, three out of nine cups (red circles) contained a mealworm as reward. Per animal the same three cups were rewarded over all trials.

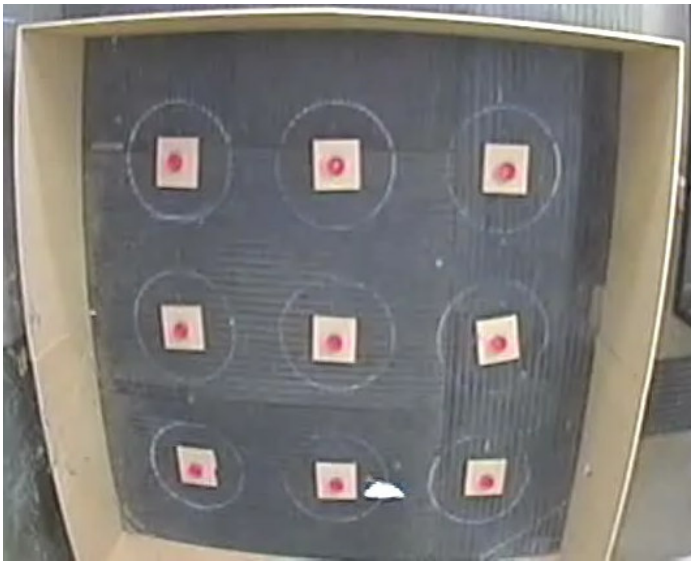

Fig. 5. Chicken in Holeboard experiment

Table 1. Definitions of measured and calculated parameters in the hole-board test.

Measure	Description
Visit of cup	When the chicken was in the circle around the cup with both feet.
Latency to visit first cup	Time elapsed between putting the chicken on the starting point and the first visit of a cup.
Trial duration	Time elapsed to find all baits, or if the chicken did not find all baits, the maximum trial duration.
Cups visited	Order and total number of cups visited
Total number of visits	Sum of all visits and revisits
Frequency visits baited set	Total number of visits to baited set.
Frequency visits never baited set	Total number of visits to never baited set.
Frequency revisits baited set	Total number of revisits to the baited set.
Frequency revisits never baited set	Total number of revisits to the never baited set.
Number of mealworms eaten	Number of mealworms eaten at the end of a trial. Not to be confused with frequency of visits to baited set.
Working memory	Working memory is calculated as number of food rewarded cup visits divided by number of visits to baited set of cups (number of mealworms eaten) / (number of visits and revisits to cups of the baited set).
Reference memory	Reference memory is calculated as number of visits to baited set of cups divided by total number of cup visits. (number of visits and revisits to the baited set) / (number of visits and revisits to all cups).

Voluntary Approach Test

At 26 days of age the chickens were individually subjected to a voluntary approach test (VAT). The VAT arena measured 2.44 m x 2.44 m. A chicken was placed in the far left corner. After one minute an experimenter entered the arena in the lower right corner and kneeled down presenting food in his hand. Latency to peck at food in the experimenters hand was measured. A trial had a time limit of 2 minutes. The sessions were recorded with a camera that was attached to the ceiling (The Observer software package, Noldus Information Technology B.V., Wageningen, The Netherlands). Testing order was randomized (SAS PLAN procedure). Data was analyzed using a non-parametric Mann-Whitney U-test (two independent samples, asymptotic probability, two-tailed).

Results

Mineralocorticoid Receptor Expression

Figure 6. shows how the mouse monoclonal antibody was able to detect chicken MR (at ~110kDa). In mHp, DM and NCL+PoA mean color intensity (Fig. 7) was measured per hemisphere (L or R), mean color intensity both hemispheres (MeanL+R) and differences as absolute values ($|AbsL-R|$). Low values indicate high expression, high values indicate low expression (e.g. black = 0, white/no color=200). All statistics are reported in Appendix I and summarized here. Brooding hen presence lead to increased MR expression in the NCL+PoA (Fig. 8.a.; AbsL-R brooding 9.197±1.170; AbsL-R no-brooding 5.671±0.729, $p<0.05$), and a trend to increased MR levels in the left hemisphere in the NCL+PoA (Fig. 8.b.; $p=0.051$). No differences in NCL + PoaA MR expression were seen between selection lines. No statistical differences were revealed in mHp and DM.

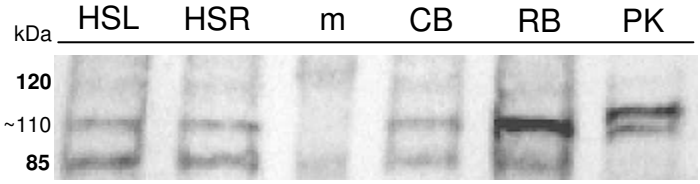

Fig. 6. Western blot analysis of MR in chicken brain and pig kidney. Mouse monoclonal MR antibody (1:500, Abcam) revealed multiple bands ranging from ~20-150 kDa (m is marker). MR is found at ~110kDa from tissue homogenates of adult chicken left hemisphere (HSL), right hemisphere (HSR), cerebellum (CB), rest of brain material (RB) and pig kidney lysate (PK, positive control).

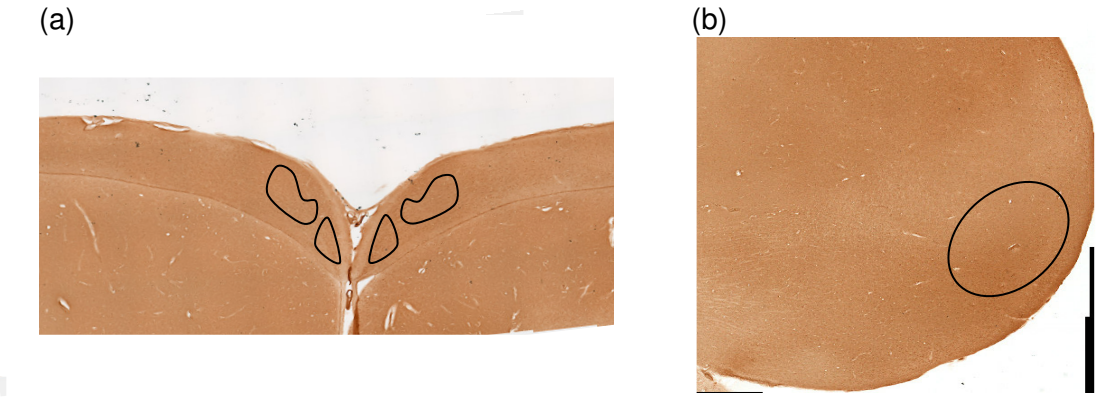

Fig. 7. 3'3-Diaminobenzidine staining of the analyzed hippocampal areas (a) and nidopallium caudolaterale + posteriosis amygdalopallii (b).

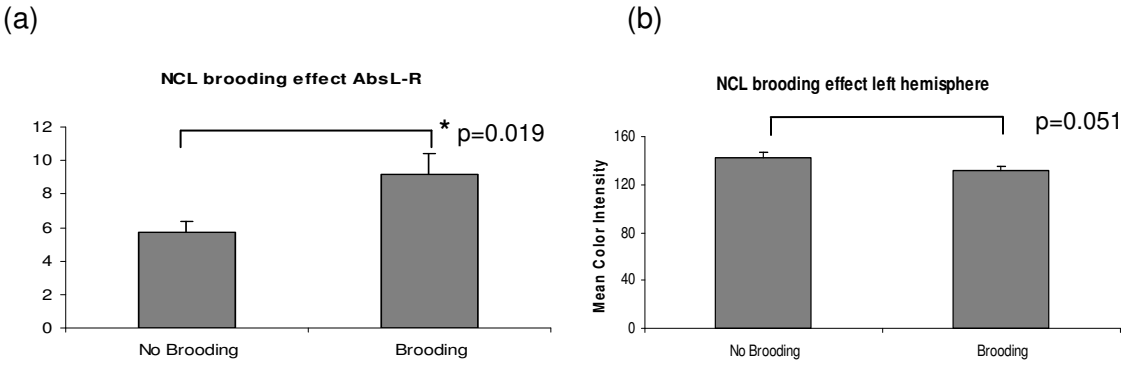

Fig 8. NCL+PoA AbsL-R values [Mean ± standard error of the mean (SEM)] for brooding effect (a) and brooding effect on the left hemisphere NCL+PoA (b).

Open-field test

There were no significant differences in the total number of distress calls between the selection lines (Fig. 9.a). No significant differences between lines were found in latency to walk, total time spent walking and total distance walked (Fig. 9 b-d).

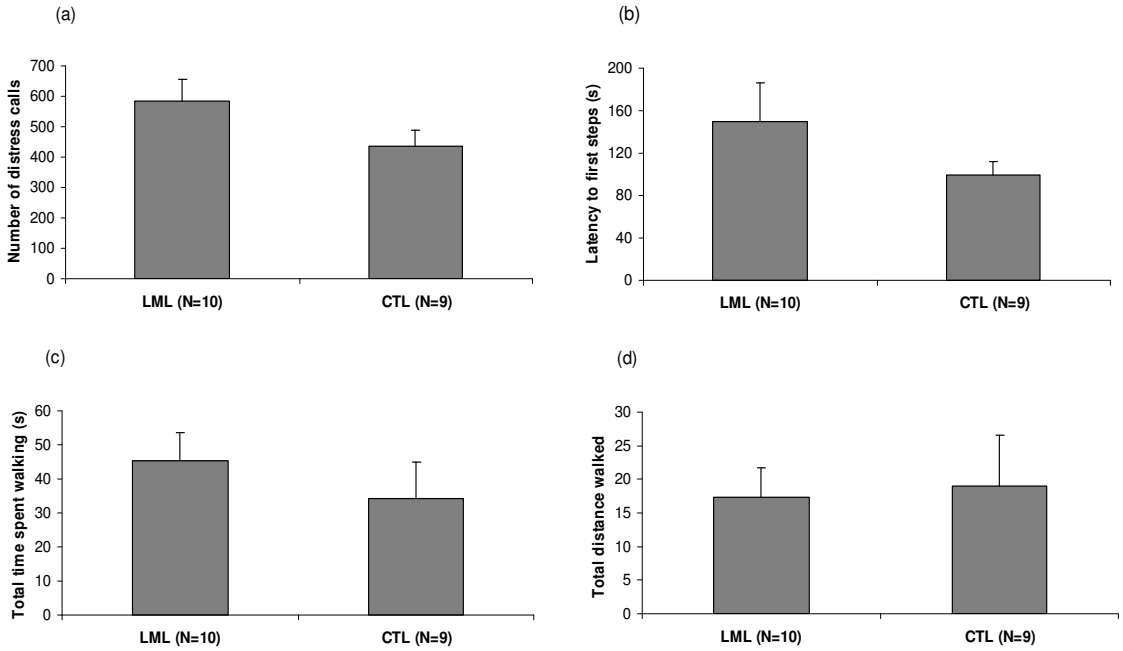

Fig. 9. Number of distress calls (a), latency to first steps (b), total time spent walking (c) and total number of line crossings (d) in the open-field test at 1 week of age in chicks from control (CTL) and low mortality line (LML). The means ± standard error of the means (SEM)] are depicted per line.

T-maze

The 12- to 15-day-old LML chickens spent more time with conspecifics (Fig. 10.c; LML:181±38s; CTL:0±0s $p<0.01$) in the T-maze (see Figure 10.b). The LML needed less time to find conspecifics (not analyzed statistically, as CTL showed ceiling effect). The chickens in the LML showed a significant learning curve over the first four days in the time needed to find conspecifics in the T-maze ($F_{3,27}=3.67$; $p<0.05$). Analysis of the orthogonal trend components revealed that there was a tendency to a linear decrease in time to find conspecifics ($t_9=7.69$, $0.1>p>0.05$). The third order trend deviated from ($t_9=7.69$,, $p<0.0001$ for cubic trend) indicating that this variable showed a sigmoid development over days.

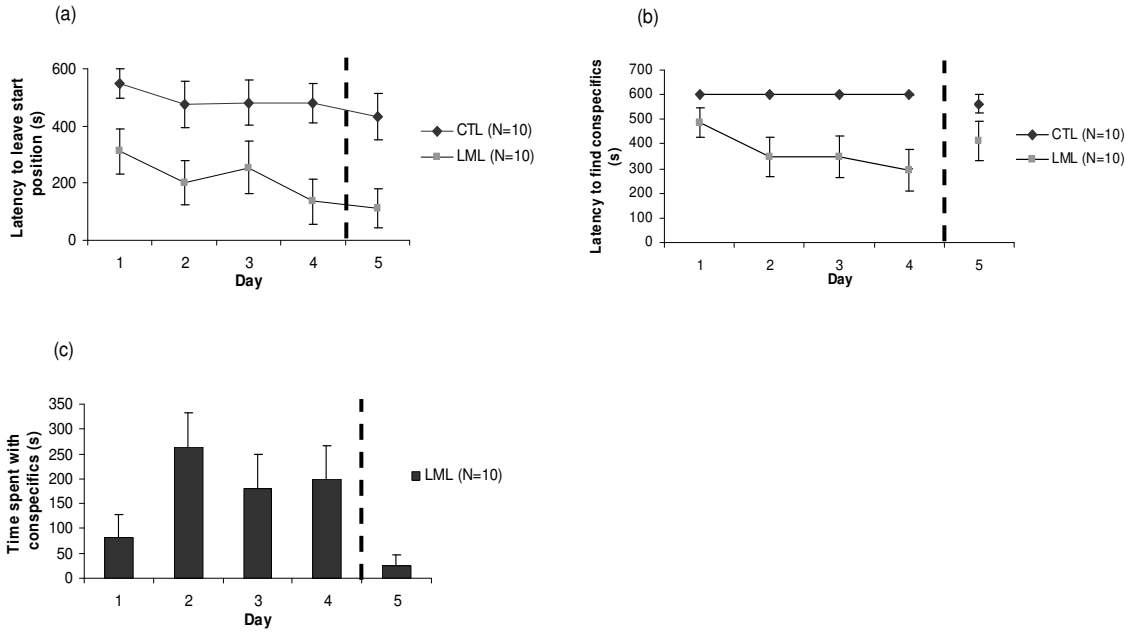

Fig. 10. LML and CTL T-maze results on latency to leave start position (a), latency to find conspecifics (b) and time spent with conspecifics (c) over five testing days in T-maze to test sociality [Mean ± standard error of the mean (SEM)]. Day 1-4 conspecifics were present in setup, day 5 conspecifics were absent.

Holeboard

No line differences were found for the latency to walk, trial duration, reference memory (fig. 11 a) and working memory (fig. 11 b) in the holeboard over 12 sessions in 6 testing days (2 session per testing day) (results not shown).

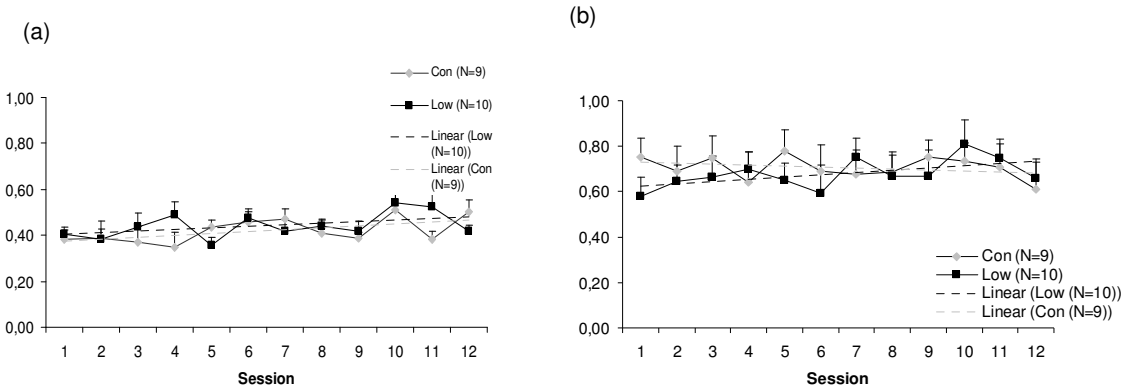

Fig. 11. Holeboard results [Mean ± standard error of the mean (SEM)] for reference memory (a) and working memory (b)

Voluntary Approach test

At 26 days the LML had a significantly shorter latency to approach a familiar human experimenter in the voluntary approach test (LML: 17±6s; CTR: 84±14s; Mann-Whitney U=9.5, $p<0.005$).

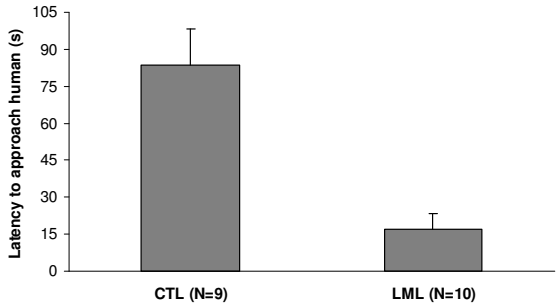

Fig. 12. Latency [Mean ± standard error of the mean (SEM)] to approach human in the voluntary approach test at 26 days of age in chicks from control (CTL) and low mortality line (LML).

Discussion

An aim of this study was to investigate the effects of selection on low mortality on sociality and fearfulness of young White Leghorn chicks. The young LML chicks showed more sociality and were less fearful compared to their CTL counterparts. Immunocytochemistry results for the effects of selection on low mortality and of brooding by a mother hen on MR expression in 40 weeks old White Leghorn laying hen hippocampus and NCL+PoA showed that brooding hen presence lead to increased lateralization of MR expression in the NCL+PoA. Together with the trend of increased MR levels in the left hemisphere in the NCL+PoA, this indicates that brooding hen presence may facilitate lateralization of the chicken NCL+PoA. The area measured consisted of the ventral part of the NCL and the dorsal part of the nucleus posteriosis amydalopallii. New analysis of PoA and NCL separately will be performed in the near future to determine if the lateralization of MR expression in the NCL+PoA is due to increased MR expression in the NCL, PoA or in both limbic areas.

As there was no line difference in MR lateralization we assume that the chicks brain plasticity during the rearing period is susceptible to brooding hen influences on the development of MR expression in the NCL+PoA seen at adult age. Vallortigara and Rogers (2005) describe how steroid hormonal mechanisms, such as corticosterone

levels in the embryo during the final stages before hatching, affect the alignment of lateralization. The present finding may give us a strong lead that stress related hormones, such as corticosterone, also after hatching may facilitate lateralization during the rearing period when the chick brain is still growing. The allostatic load, costs paid by the body after prolonged and continued adaptation to chronic stress, could then well be an explanation of the differences found between the brooded and non-brooded chicks. The absence of the brooding hen may act as chronic stressor. This stress affects continuous imbalance of glucocorticoids leading to allostatic load (McEwen 2006) which affects the structural plasticity of the brain. McEwen found a negative effect of chronic psychosocial stress on the neurogenesis in rodent hippocampus, amygdala and prefrontal cortex, an observation that is in line with our findings that brooding affects MR expression in the NCL and PoA of laying hens. Allostatic load may also be related to the glucocorticoid cascade hypothesis, also named the neurotoxicity hypothesis that suggests a relationship between cumulative exposure to high glucocorticoid levels and hippocampal atrophy (Lupien et al. 2009). Reduced maternal care has been demonstrated to affect CRH binding sites density in the prefrontal cortex and amygdala, as well as in the hypothalamus, hippocampus and cerebellum (Lupien et al. 2009).

Lupien et al. (2009) also describes in her review how early-life events affect the brain, behavior and cognition throughout the lifespan. Relevant to the present study is the finding that prenatal stress and reduced maternal care in rats cause decreased numbers of MR and GR in the hippocampus at juvenile and adult age. Our study showed that brooding hen presence during the first 6 weeks was the factor leading to left hemisphere NCL+PoA lateralization visible at 40 weeks. Brooding hen presence can thus be seen as an early-life factor with effects that extend into adult age.

Brooding hen presence as an early-life event has an epigenetic effect on lateralization as it leads to phenotypical changes, but does not alter the genotype (McEwen 2008). Vallortigara and Rogers (2005) extensively discuss lateralization at population and individual level as an evolutionarily stable strategy (ESS) under social pressure when the individual has to deal with other asymmetrical individuals. ESS is a term best used on wildlife populations where the best strategy is most profitable for the population survival rate. In the selection on low mortality experiments the population survival rate is strongly influenced by human interaction.

The selection experiment may lead to advantageous lateralization with beneficial consequences. The selection for low mortality acts as an indirect selection of lateralized

individuals, with low mortality as outcome. Vallortigara and Rogers (2005) describe how certain functions are lateralized in several species. For birds or chicks they describe lateralized functions in the left hemisphere for foraging with discrimination and/or manipulation of food items, inhibition of aggression, recognition of categories/attention to large changes, recognition of species-typical vocalizations, attention to landmarks and attention to local cues. Right hemisphere lateralized functions are fear, aggression, recognition of individual conspecifics, spatial cognition and attention to global cues. In our opinion left hemisphere lateralization is more preferable as the before mentioned functions of the left hemisphere are more closely related to the propensity of feather pecking, e.g. foraging with discrimination and/or manipulation of food items.

The MR is involved in the neuroendocrine and behavioral responses to stressors by promoting adaptation to stress, neuronal integrity and stable excitatory tone, cognitive processes and flexibility in selection of appropriate behavioral responses (Oitzl et al. 2009; Joëls et al. 2007). In modern poultry husbandry chickens are not able to show natural foraging behavior, leading to redirected pecking behavior at conspecifics, severe feather pecking and cannibalism. Better behavioral flexibility in LML by increased MR expression could be one of the factors underlying decreased feather pecking and cannibalism in LML chickens, as the behavioral flexibility facilitate that LML chickens can better cope and adapt to the impaired possibility to show natural foraging behavior. However, in this study we did not find a selection response on MR lateralization. The immunocytochemistry staining with 3'3-Diaminobenzidine gave a rather high background and the Western blotting with the mouse monoclonal MR antibody revealed several bands on the blot. This indicates that both laboratory techniques were not highly specific. Optimizing the laboratory techniques might give more clear results, especially the immunocytochemistry, about MR expression and MR lateralization in the brain. Future pharmacological experiments may also emphasize the role of the MR in behavioral flexibility.

This study was also performed to investigate the effects of selection on low mortality on fearfulness and sociality in young chicks. Rodenburg et al. (in press 2009) found in open-field tests line differences in fearfulness between 5-6 week old LML and CTL chickens. LML chickens had a stronger social motivation and walked more in the open-field test than the CTL chickens. We did not observe this in 1 week old chicks as we

could not find any differences between the two lines in the open-field test. This may be due to the small number of birds used in the present study. However, at 12-16 days the LML spent more time with conspecifics and showed a significant learning curve in the amount of time to find conspecifics in the T-maze, whereas the control line did not show a learning curve. Increased time spent with conspecifics means better sociality and social motivation (Boers 2009; Jones et al.1999). This indicates that selection on low mortality leads to chickens with an increased sociality. The observation of increased sociality or social motivation is strengthened by the observation that on the fifth day of the T-maze experiments, when no conspecifics were present in the T-maze, the LML showed much less motivation to visit the compartment where the conspecifics were during the first 4 testing days, and the increased latency to leave the starting position on day 5 compared to the first 4 testing days.

At 26 days of age the LML had a shorter latency to approach the experimenter in the voluntary approach test. This indicates that young chicks of the LML are less fearful than their CTL counterparts. Bolhuis et al. (2009) showed similar results in a sudden human approach test in 42 week old LML and CTL birds. The increased sociality and reduced fearfulness of the LML line are likely to underlie the reduced development of the maladaptive behaviors feather pecking and cannibalism in adult life, i.e. the LML chickens may be better prepared to adapt to the challenges and stressors of living in group housing.

We did not see a line effect on the working and reference memory in the holeboard, thereby failing to show line differences in cognitive learning and spatial. As this was the second time that the spatial holeboard was used on chickens more experiments need to prove that the holeboard task is suited to assess spatial discrimination learning in laying hens.

Conclusion

Selection on low mortality for four generations leads to increased sociality and reduced fearfulness in young White Leghorn laying hens. We suggest that these beneficial behavioral characteristics may be a good indicator that those chickens may have a reduced propensity to develop feather pecking and cannibalism at adult age. Brooding by a mother hen may lead to reduced feather pecking and

cannibalism due to lateralization of MR expression in the nidopallium caudolaterale, the posterior amydalopallii or both these areas. Both selection on low mortality and the presence of a brooding hen during the rearing period may contribute to increased welfare in laying hens. Future attention should be aimed at implementing the group selection and early-rearing conditions in larger breeding programs.

The T-maze and Voluntary Approach tests seem to be suitable to test sociality and fearfulness in young chicken behavioral assessments.

Acknowledgements

I would like to thank Hendrix Genetics for providing the birds. The Animal Breeding and Genomics Centre, Wageningen University and Research Center is acknowledged, especially Dr. Ir. Bas Rodenburg, for their cooperation and support on this project. The people of Animal, Welfare and Society of Utrecht University are acknowledged for their technical support in the MR analysis. I would like to thank Dick van de Ploeg, Wim van Brenk and Patricia Gadella of the 'Tolakker'. I would like to thank Elly Zeinstra for her technical assistance. I'm thankful that Dr. habil Franz Josef van der Staay offered me the opportunity to do this study in his group. A special thanks goes out to Dr. Rebecca Nordquist for being an excellent supervisor.

References

Arts J.W.M., F.J. van der Staay, E.D. Ekkel. Working and reference memory of pigs in the spatial holeboard discrimination task. Behav Brain Res. 2009. 4;205(1):303-6

Blokhuis H.J. Feather-pecking in poultry: its relation with ground-pecking. Appl. Anim. Behav. Sci., 1986. 16:63-67

Boers, M. Assessment of emotional reactivity, learning and memory in chickens -T-maze & Y-maze. 2009. Online version available via repository Igitur, Library University Utrecht:http://igitur-archive.library.uu.nl/student-theses/2009-1125-200122/UUindex.html

Bolhuis J.E., E.D. Ellen, C.G. van Reenen, J. de Groot, J. ten Napel, R.E. Koopmanschap, G. de Vries Reilingh, K.A. Uidehaag, B. Kemp, T.B. Rodenburg. Effects of genetic selection against mortality on behavior and peripheral serotonin in domestic laying hens with trimmed and intact beaks. Physiol. & Behav. 2009. 97: 470-475

Braun K., E. Lange, M. Metzger, G. Poeggel. Maternal separation followed by early social deprivation affects the development of monoaminergic fiber systems in the medial prefrontal cortex of Octodon degus. Neurosc. 2000. 95: 309-318

Brinks V., S. Berger, P. Gass, E.R. de Kloet, M.S. Oitzl. Mineralocorticoid receptors in control of emotional arousal and fear memory. Horm. And Behav. 2009. 59:232-238

Cerqueira J.J., F. Malliet, O.F. Almeida. T.M. Jay, N. Sousa. The prefrontal cortex as a key target of the maladaptive response to stress. The J. of Neurosc. 2007. 27: 2781-2787

Daisley J.N., E. Mascalzon, O. Rosa-Salva, R. Rugani, L. Regolin. Latereralization of social cognition in the domestic chicken (Gallus gallus). Phil. Trans. B. 2009. 364-965-981

Diamond M.C. Response of the brain to enrichment. Anais da Academia Brasileira de Ciências. 2001. 73: 212-220

Ellen E.D., W.M. Muir, F. Teuscher, P. Bijma. Genetic improvement of traits affected by interactions among individuals: sib selection schemes. Genetics. 2007. 176: 489-499

Forkman B., A. Boissy, M.-C. Meunier-Salaün, E. Canali, R.B. Jones. A critical review of fear tests used on cattle, pigs, sheep, poultry and horses. Physiol.&Behav. 2007. 92: 340-374

Frick K. and S.M. Fernandez. Enrichment enhances spatial memory and increases synaptophysin levels in aged female mice. 2003. Neurobiology of Aging. 24: 615-626

Griffing B. Selection in reference to biological groups. I. Individual and group selection applied to populations of unordered groups. Aust. J. Biol. Sci. 1967. 20: 127-139.

Gruss M. and K. Braun. Distinct activation of monoaminergic pathways in chicken brain in relation to auditory imprinting and stressful situations: a microdialysis study. Neuroscience. 1997. 76(3): 891-899

Harlow H.F. and M. Harlow. Learning to love. American Scientist. 1966. 54: 244-272

Joëls M., H. Karst, R. DeRijk, E.R. de Kloet. The coming out of the brain mineralocorticoid receptor. TRENDS in Neurosciences. 2007. Vol.31.No.1:1-7

Jones R.B., H.J. Blokhuis, G. Beuving. Open-field and tonic immobility responses in domestic chicks of two genetic lines differing in their propensity to feather peck. Brit. Poul. Sci. 36: 525-530

Jones R.B., R.H. Marin, D.A. Garcia, A. Arce. T-maze behaviour in domestic chicks: a search for underlying variables. Anim. Behav. 1999. 58: 211-217

Kuenzel W.J. and M. Masson. A stereoraxic atlas of the brain of the chick (*Gallus domesticus*). 1988. ISBN 0-8013-3700-6

Lupien S.J., B.S. McEwen, M.R. Gunnar, C. Heim. Effects of stress trhoughout the lifespan on the brain. behaviour and cognition. Nature. 2009. 10:434-445

Manteca X. Neurophysiology and assessment of welfare. Meat Sci. 1998. 48:205-218

McEwen B.S. Plasticity of the hippocampus: Adaptation to chronic stress and allostatic load. 2006. Ann. New York Ac. of Sc. 933:265-277

McEwen B.S. Understanding the potency of stressful early life experiences on brain and body function. 2008. Metab. Clin. And Exp. 57”S11-S15

Mirescu C.and E. Gould. Stress and adult neurogenesis. Hippocampus. 2006. 16:233-238

Nicol C. How animals learn from each other. Appl. Anim. Beh. Sc. 2006. 100:58-63

Oitzl M.S., D.L. Champagne, R. van der Veen, E.R. de Kloet. Brain development under stress: Hypotheses of glucocorticoid actions revisited. NeuroSci. Biobehav. Rev. 2009. doi:10.1016/j.neubiorev.2009.07.006

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

Perré Y., A.-M. Wauters, M.-A. Richard-Yris. Influence of mothering on emotional and social reactivity of domestic pullets. Appl. Anim. Behav. Sci. 2002. 75:133-146

Riedstra, B. **and T. G. G. Groothuis**. Early feather pecking as a form of social exploration: the effect of group stability on feather pecking and tonic immobility in domestic chicks. Appl. Anim. Behav. Sci. 2002. 77:127-h138.

Rodenburg T.B., A.J. Buitenhuis, B. Ask, K.A. Uitdehaag, P. Koene, J.J. van der Poel, J.A.M. van Arendonk, H. Bovenhuis. Genetic and phenotypic correlations between feather pecking and open-field response in laying hens at two different ages. Behav. Gen. 2004. 34(4):407-415

Rodenburg T.B., H. Komen, E.D. Ellen, K.A. Uitdehaag, J.A.M. van Arendonk. Selection method and early-life history affect behavioural development, feather pecking and cannibalism in laying hens: A review. Appl. Anim. Behav.Sci.2008. 110(3-4):217-228

Rodenburg T.B., J.E. Bolhuis, R.E. Koopmanschap, E.D. Ellen, E. Decuypere. Maternal care and selection for low mortality affect post-stress corticosterone and peripheral serotonin in laying hens. Physiol Behav. 2009. 98(5):519-23.

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

Rogers L.J., P. Zucca, G. Vallortigara. Advantages of having a lateralized brain. Proceeding of the Royal Society of London. Series B: Biological Sciences. 2004. 271: S420-S442

Römkens, D.M.J.M. *Assessment of emotional reactivity, learning and memory - open field & hole-board*. 2009. Online version available via repository Igitur, Library University Utrecht:http://igitur-archive.library.uu.nl/student-theses/2009-1125-200120/UUindex.html

Saint-Dizier H., P. Constantin, D.C. Davies, C. Leterrier, F. Lévy, S. Richard. Subdivision of the arcopallium/posterior pallial amygdala complex are differentially involved in the control of fear behaviour in the Japanese quail. Brain Research Bulletin. 2009. 79: 288-295

Vallortigara G. and L.J. Rogers. Survival with an asymmetrical brain: Advantages and disadvantages of cerebral lateralization. Behav. And Brain Sc. 2005.28:575-633

Van Praag H., G. Kempermann, F.H. Gage. Neural consequences of environmental enrichment. Nature Rev. Neurosc. 2000. 1: 191-198

Vestergaard K.S and L. Lisborg. A model of feather pecking development which relates to dustbathing in the fowl. Behaviour. 1993. 126: 291-308

[1]http://avianbrain.org/nomen/Chicken_Atlas.html

[2]http://avianbrain.org/new_terminology.html

Appendix I

		CTL-NB	CTL-B	LML-NB	LML-B	Brooding		Line		Brooding x Line	
Area	Measured	AVE ± s.e.m	AVE ± s.e.m	AVE ± s.e.m	AVE ± s.e.m	F	Sig.	F	Sig.	F	Sig.
NCL + Ai	Left	142,1 ± 6,5	135,0 ± 5,0	143,5 ± 6,0	127 ± 3,7	4,252	0,051	0,331	0,571	0,667	0,423
	Right	143,4 ± 5,0	141,3 ± 5,9	147,8 ± 6,0	133,5 ± 4,3	2,123	0,159	0,095	0,761	1,193	0,287
	mean Left_Right	142,7 ± 5,6	138,1 ± 5,4	145,6 ± 5,9	130,2 ± 3,9	3,236	0,086	0,204	0,656	0,945	0,342
	AbsL-R	6,3 ± 1,3	9,3 ± 0,8	5,0 ± 0,7	9,1 ± 2,7	6,372	**0,019***	0,297	0,591	0,181	0,675
mHp	Left	131,6 ± 7,8	131,2 ± 9,1	138,1 ± 8,1	129,5 ± 5,5	0,518	0,480	0,007	0,934	0,442	0,514
	Right	133,9 ± 7,3	132,1 ± 8,3	136,1 ± 6,3	124,4 ± 8,3	0,656	0,427	0,100	0,756	0,382	0,543
	mean Left, Right	132,9 ± 6,9	131,6 ± 8,7	137,2 ± 7,0	128,8 ± 6,5	0,629	0,437	0,014	0,908	0,422	0,523
	AbsL-R	5,8 ± 0,8	4,8 ± 1,2	8,2 ± 1,2	4,6 ± 1,8	3,433	0,079	0,809	0,379	1,130	0,300
DM	Left	132,6 ± 6,5	127,5 ± 9,4	135,3 ± 6,9	142,1 ± 14,4	0,008	0,929	0,738	0,400	0,352	0,560
	Right	133,7 ± 6,2	129,0 ± 8,1	132,3 ± 5,4	142,6 ± 22,4	0,038	0,848	0,384	0,543	0,502	0,487
	mean Left, Right	133,4 ± 5,8	129,1 ± 8,1	133,8 ± 5,9	142,3 ± 16,4	0,022	0,885	0,545	0,469	0,436	0,517
	AbsL-R	5,5 ± 1,6	5,6 ± 2,1	8,6 ±1,6	8,0 ± 2,8	0,008	0,929	1,941	0,179	0,031	0,861