The Effects of the Alteration of the Phenotypic Appearance and Modifications of the Early Environment on the Welfare of Laying Hens.

Irene Campderrich Lecumberri

Doctoral Thesis

Universidad del País Vasco
Euskal Herriko Unibertsitatea

Als meus pares,

Ramón i Maria Jesús,

gràcies per haver-me ensenyat a estimar la vida,

Us estimo,

The Effects of the Alteration of the Phenotypic Appearance and Modifications of the Early Environment on the Welfare of Laying Hens.

International PhD Thesis submitted by:

Irene Campderrich Lecumberri

Doctoral Thesis submitted to the:

Department of Zoology and Animal Cellular Biology,
Faculty of Science and Technology,
University of The Basque Country.

Thesis directed by: Dra. Inmaculada Estévez

Neiker, Department of Animal production
IKERBASQUE, Basque Foundation for Science

Vitoria-Gasteiz, March 2021

TABLE OF CONTENTS

List of figures

Figure 3.1 *Photo description of the two PA treatments used in this experiment. M (presenting Marked individuals), U (presenting Unmarked individuals).*

Figure 3.2 *Total aggression (interactions per bird/40 min). Frequency of total aggression per bird at T0 (27–28 weeks; 3.2A), T1 (35–36 weeks; 3.2B)), T2 (39–40 weeks; 3.2C) and T3 (45–46 weeks; 3.2D). Bars represent means ± SE. M = marked; U = unmarked. Phenotypic appearance (PA) treatments: originally homogeneous (100U, 100M) and controls (30M/70U, 50M/50U, 70M/30U). Different letters denote significant differences among PA treatments at P<0.05.*

Figure 3.3 *Changes in total aggression (interactions per bird/40 minutes) for each phenotypic appearance (PA) treatment across time. Changes in total aggression per bird for each PA treatment; originally homogeneous groups (100U, 100M) and controls (30M/70U, 50M/50U, 70M/30U), across time periods: T0 (27–28 weeks), T1 (35–36 weeks), T2 (39–40 weeks) and T3 (45–46 weeks). Different letters denote significant differences across time (P<0.05).*

Figure 3.4 *Directionality of aggressive interactions across time. M = marked; U = unmarked. Differences between observed and expected aggressive interactions (means ± SE) for each possible interacting pair (MM, MU, UM and UU) and phenotypic appearance (PA) treatment: originally homogeneous (100U, 100M), and controls (30M/70U, 50M/50U, 70M/30U). 3.4A) T0: 27–28 weeks; 3.4B) T1: 35–36; 3.4C) T2:39–40; 3.4D) T3:45–46.*

Different letters indicate significant differences among interacting pairs within the same PA treatment.

Figure 3.5 *Directionality of aggressive interactions at T3. M = marked; U = unmarked. Differences between observed and expected aggressive interactions (means ± SE) at T3 for each possible interacting pair (MM, MU, UM and UU) and phenotypic appearance (PA) treatment: originally homogeneous (100U, 100M) and controls (30M/70U, 50M/50U, 70M/30U), according to group size (GS 10, 20 or 40 birds). Different letters indicate significant differences, within each GS and PA treatment, for each type of interacting pair (P<0.05)*

Figure 4.1 *Complex versus Simple environments. Photo of two pens from the current experiment; the one on the left side represents the Complex Environment (CENV) treatment provided with a drinker, a feeder, wood shavings, three wooden blocks, four perches at different levels and a hide area; while the one on the right side represents the Simple Environment (SENV) treatment, provided only with a drinker, feeder and wood shavings.*

Figure 4.2 *Experiment description. (A) Experimental design: for this experiment 96 domestic fowl chicks (Bovan robust, a white laying strain) were randomly assigned to either a Complex or a Simple home environment (CENV, SENV respectively; N=48 per treatment). Additionally, birds from each environment were allocated to either a Cold Stress (CSTR) or No Stress (NSTR) treatment. At four weeks of age birds with different early life experiences (CSTR-CENV, CSTR-SENV, NSTR-CENV, NSTR-*

SENV, two pens/treatment, eight pens in total) were exposed to an Intermittent Stressful Challenges Protocol (ISCP); (B) Distribution of the treatments across the experimental room: each square division represents a pen, F indicates the feeder, W the drinker, CENV refers to Complex Environment, SENV to Simple Environment; NSTR refers to a No Stress group while CSTR refers to a Cold Stress group.

Figure 4.3 *Chronogram of the study. Body weight (BW) was measured the first day of the study in order to balance groups accordingly and every week afterwards; birds were either assigned to a Complex Environment (CENV) or to a Simple Environment (SENV) as their rearing system; on day two of age, half of the birds from each environment were submitted to either a Cold Stress (CSTR) or a No Stress (NSTR) treatment; behavioural observations (B) were performed two times per week except for week four when the Intermittent Stressful Challenges Protocol (ISCP) was applied. The time of the measure of each immunological parameter (smears for accounting the heterophil/lymphocyte ratio (H/L ratio), Phytohemagglutinin-P injection (PHA-P) and Sheep Red Blood Cells injection (SRBC)) are indicated by arrows in the chronogram; at the end of the study we also collected tarsus weight, tarsus thickness and calculated body condition and Relative Fluctuating Asymmetry (RFA).*

Figure 4.4 *Internal body temperature for Cold Stress (CSTR) No Stress chicks (NSTR). Mean (± SE) internal body temperature for acute CSTR and NSTR chicks; temperature was registered in ºC every 2h, at 0 (beginning of the cold stress), 2h, 4h, and 6h; $^{(A\text{-}C)}$ letters indicate differences over time within the CSTR or NSTR* *treatment; $^{(a-b)}$ letters indicate differences between CSTR and NSTR birds at the same time point.*

Figure 4.5 *Number of animals feeding during application of the intermittent stressful challenges protocol (ISCP). Mean (± SE) number of animals feeding before (30, 15 min), during (0, 15, 45 and 60 min) and after (75 and 90 min) exposure to heavy metal music reproduced at 90Db; $^{(A-D)}$ letters indicate differences across time.*

Figure 4.6 *Increase in body weight estimated between two consecutive weeks. $^{(A-E)}$ letters indicate significant differences across time within each CENV or SENV treatment (p<0.05); there were no significant differences between CENV/SENV treatments at the same age period.*

Figure 4.7 *Heterophil/lymphocyte ratio evolution according to the different treatments. Mean (± se) Heterophil/lymphocyte (H/L) ratios in Cold Stress (CSTR) and No Stress (NSTR) birds kept in the Simple Environment (SENV) and a Complex Environment (CENV); NSTR treatment is represented by white bars, while CSTR by grey bars; SENV is represented by bars with a solid colour and CENV by bars with dots; $^{(a-b)}$ letters indicate differences between treatments at each sampling day and $^{(A-C)}$ letters indicate differences between the four different sample points; (T1) 2 days after hatching (directly after the exposure to the CSTR/NSTR treatment); (T2) 18 days after hatching; (T3) 28 days after hatching (just after the 5-days of exposure to the ISCP); (T4) 36 days after hatching (one week after the exposure to the ISCP).*

Figure 4.8 *Mean (± SE) percentage of inflammation measured after PHA-P injection.* (a-b) *letters indicate differences between different treatments at each injection time i.e. Day 7 of age (five days after Cold Stress (CSTR)/No Stress (NSTR) treatment) and Day 28 of age (after the ISCP was applied);* (A-B) *letters indicate differences across time within the same treatment; NSTR treatment is represented by white bars; CSTR by grey bars; SENV is represented by bars with a solid colour; CENV by bars with dots.*

Figure 4.9 *Mean (± SE) frequency of birds performing each behaviour by week.* (a-c) *letters indicate significant differences across weeks for eating, drinking, foraging, preening, resting, walking and standing.*

Figure 4.10 *Use of different areas in the complex environment treatment; Mean (+ SE) frequency of birds using the different areas of the pen by week in the CENV treatment;* (a-d) *letters indicate differences in the use of the structures (P<0.05) across weeks.*

Figure 4.11 *Use of the platform over the hide area for CSTR and NSTR birds. Mean (+ SE) frequency of birds using the platform over the hide area for the Cold Stress (CSTR) and No Stress (NSTR) treatments across different weeks;* (a-b) *letters indicate differences within each treatment across weeks; no differences among treatments were observed in post hoc comparisons regarding each observed week.*

Figure 4.12 *Use of perches by CSTR and NSTR birds. Mean (+ SE) frequency of birds perching for the Cold Stress (CSTR) and No Stress (NSTR) treatments across different weeks;* $^{(a\text{-}b)}$ *letters indicate differences within each treatment across weeks; no differences among treatments were observed in post hoc comparisons regarding each observed week.*

List of tables

Preface

The Thesis presented is submitted in order to comply with part of the requirements to obtain the Degree of Philosophiae Doctor (PhD) at the Department of Zoology and Animal Cellular Biology of the University of the Basque Country UPV/EHU (Leioa) under the PhD Program of Biodiversity, Functionality and Ecosystem Management.

The first part (Chapters 2 and 3). of the work presented in this document has been carried out at the experimental poultry facilities of the Department of Animal Production at Neiker (Arkaute, Spain). The second part (Chapter 4), was performed at the experimental facilities from the Centre of Excellence for Animal Welfare in Sweden (Swedish University of Agricultural Sciences SLU).

The present document is organized into Chapters that are described below.

Part I: General context

> **Chapter 1** covers the general introduction of the work and provides an overview of the Thesis. The aims of the Thesis, the Contributions, and the Scientific Outputs derived from the research are included in this section.

Part II: Social challenges

> **Chapter 2** includes the first study that focus on the effects of different degrees of diversity in the social groups that was obtained by manipulating the phenotypic appearance

(PA) combined with the effects of group size (GS) from day one until the pre-laying period. We explored the influence of the PA over the amount and direction of social interactions both aggressive and affiliative. We additionally tested if those effects were dependent on the GS and the frequency of the phenotype.

Chapter 3 includes the second study performed and explores the effects of the alteration of the phenotypic appearance (PA) in originally homogeneous groups of laying hens during adulthood. In this case, we explored the effects of sequentially altering the PA of different proportions of birds within the group on aggression. We additionally explored whether those effects where GS and frequency dependent.

Part III: Environmental context effects

Chapter 4 includes the third study performed and explores whether the provision of a complex environment may help birds to cope better with early stressors or better prepare them to future challenges. This experiment was based on the measure of different morphological, behavioural and immunological variables.

Part IV: Discussion and Conclusions

Chapter 5 includes the General Discussion and Conclusions. Limitations of the present study are also discussed here.

Financial support

The Thesis presented has received support by the following funding sources;

Spanish Ministry of Economy and Competitivity through the project "MODELAY: Modelling group size and phenotypic appearance through game theory for the optimization of the welfare, health and performance of laying hens." Project reference: AGL2010-18276, grant reference: BES-2011-050055.

Spanish Ministry of Economy and Competitivity through the program "Ayudas a la movilidad predoctoral para la realización de estancias breves en centros de I+D Españoles y extrangeros en las convocatorias de 2014 y 2015 (grant references: EEBB-I-14-08369, EEBB-I-15-10297 respectively).

The Swedish Council Formas funded the Animals and Housing expenses needed for the development of the experiment realized at the Centre of Excellence in Animal Welfare Science (at the Experimental Facilities established at Lövsta).

The funding sources have not had any implications in the writing of the Thesis or of the publications generated from it.

NEIKER MEMBER OF BASQUE RESEARCH & TECHNOLOGY ALLIANCE

GOBIERNO DE ESPAÑA MINISTERIO DE ECONOMÍA Y COMPETITIVIDAD

EUSKO JAURLARITZA GOBIERNO VASCO

FORMAS

Executive Summary

Animals kept for production, such as laying hens, are normally exposed to a wide range of social and environmental challenges from an early age. The focus of the work presented was to explore the effects that the alteration of the social and environmental context had on the welfare of laying hens. This Thesis is divided into two main parts: 1) The first part (Chapters 2 and 3), aimed to investigate whether the alteration of the phenotypic appearance (PA) of different proportions of birds affected the frequency and direction of their social interactions. A second objective from this research was to determine whether those effects were group size (GS) and frequency-dependent. For these studies a total of 1050 day old chicks were randomly assigned to 45 pens of 3 different GS, 10, 20 and 40 birds (constant density 8 hens/m^2). For all GS treatments, the PA of different bird proportions was modified with a black mark at the back of their head (0, 30, 50, 70, 100% of the birds marked (M)). Consequently, for the experiment performed in Chapter 2, these were the experimental groups to be tested. In these groups, the frequency and direction of the different aggressive and affiliative behaviours performed among group members was collected by behavioural *ad libitum* observations of each pen. The potential effects of the variables mentioned over the frequency and direction of the social interactions during the prelaying period were analysed with mixed model Anovas. Chapter 3, was a follow up study, in which the effects on aggression of altering the PA in originally homogeneous groups of adult laying hens (0, 100% M)

were analysed. In this experiment, originally homogeneous groups (100% unmarked (U) and 100% marked (M)) were sequentially altered by marking, or unmarking 30, 50 and 70% of the birds at 34, 38 and 44 weeks of age respectively. Frequency and directionality of aggressive interactions in altered groups were compared with that of non-altered heterogenous controls. For this initial part of the Thesis, we found that while in adult laying hens the effects of PA were clear and profound, in pullets, the effects found were minor. Adult laying hens respond to alterations of PA escalating aggression in those altered groups and clearly directing such aggression towards those birds experimentally altered (either newly M or U). These results suggest that imprinting may play an important role during early ages regulating what pullets recognize as “normal” phenotypes depending on what they find in their close environment. However, as adults, laying hens are much more reactive to changes in their social environment. Such response may be the result of years of evolution, as aggression towards altered phenotypes may constitute a defence against invaders that may expose the population to high competition for resources, new pathogens, or other additional risks. We propose here, that phenotype matching mechanisms may regulate this defensive reaction against new phenotypes. Phenotype matching mechanisms may explain why in adult flocks of laying hens, diversity due to growth, injures or diseases, could lead to the escalation in aggressive interactions that may compromise the survival of targeted birds in commercial conditions.

The second part of this Thesis (Chapter 4), explores whether the provision of a complex environment (CENV) during early life, better prepare birds to cope with stressful events, even functioning as buffers against future unpredictable stressful episodes. In this case, 96 one day old pullets were assigned to eight pens (12 birds/pen). Half of the chicks were assigned to a Complex Environment (CENV) while the others to a Simple Environment (SENV). Additionally, half of the chicks from each of these treatments were assigned to a Non-Stress (NSTR, 33 ºC) or to acute Cold Stress (CSTR 18-20ºC) treatment during six hours on their second day of life. At four weeks of age, chicks from those four different backgrounds were assigned to an Intermittent Stressful Challenges Protocol (ISCP).The results of this experiment indicate that in an immunological test indicative of pro-inflammatory status phytohemagglutinin-P (PHA-P challenge), the response of CSTR birds was ameliorated by rearing chicks in a CENV as they showed similar response to NSTR and a significantly better proinflammatory response than CSTR birds reared in a CENV (five days after CSTR was applied). A similar better response when coping with new challenges (ISCP) was observed in birds reared in a CENV compared to those from a SENV. Birds reared in a CENV showed lower heterophil/lymphocyte ratio after the ISCP than birds reared in SENV. The provision of a CENV also increased resting which may have favoured stress recovery. Additionally, exposure to CSTR at an early age might have rendered birds more vulnerable to future stressful challenges. CSTR birds show lower humoral immune responses (against sheep red blood cells (SRBC)) after the ISCP and started using elevated structures in the CENV later compared to their NSTR conspecifics. Our study suggests that environmental complexity during rearing may not only help to ameliorate the consequences of early stressful events, but may also help birds to cope better with future challenges. This, in turn, reflects the importance of early provision of a CENV in commercial conditions which may help to reduce the negative stress related effects.

In conclusion, this Thesis provides important information regading the coping response of the birds to different social and environmental challenges. The results obtained can help us to better design production environments and management protocols for improving the welfare of laying hens. These results also suggest that the possibilities and response potentiality of laying hens would be regulated by the social and physical inputs they receive along their development. These results highlight the importance of social and physical inputs along ontogeny in the regulation of coping strategies and response potentiality of laying hens.

Scientific output

The Thesis is based on the following articles, which are referred to in the text by their Roman numerals:

I. **Campderrich, I.**, Liste, G. and Estevez, I. (2017). Group size and phenotypic appearance: Their role on the social dynamics in pullets. Applied Animal Behaviour Science. 189, 41-48. https://doi.org/10.1016/j.applanim.2017.01.014

II. **Campderrich, I.**, Liste, G. and Estevez, I. (2017). The looks matter; aggression escalation from changes on phenotypic appearance in the domestic fowl. Plos One. 12(12), e0188931.

https://doi.org/10.1371/journal.pone.0188931

III. **Campderrich, I.**, Nazar, F.N., Wichman, A., Marin, R.H., Estevez, I. and Keeling, L.J. (2019). Environmental complexity: a buffer against stress in the domestic chick. PloS one, 14(1), e0210270.

https://doi.org/10.1371/journal.pone.0210270

Additional scientific articles written during the course of my doctoral studies, but not included in the Thesis:

- Zidar, J., **Campderrich, I**., Jansson, E., Wichman, A., Winberg, S., Keeling, L.J., and Løvlie, H. (2018). Environmental complexity buffers against stress-induced negative judgement bias in female chickens. Scientific reports, 8(1), 5404. https://doi.org/10.1038/s41598-018-23545-6

- Frigerio, D., Cibulski, L., Ludwig, S.C., **Campderrich, I.,** Kotrschal, K., and Wascher, C. A. (2016). Excretion patterns of coccidian oocysts and nematode eggs during the reproductive season in Northern Bald Ibis (*Geronticus eremita)*. Journal of Ornithology, 157(3), 839-851. https://doi.org/10.1007/s10336-015-1317-z

- Nazar, F.N., Marin, R.H., Liste, G., **Campderrich, I**. and Estevez, I. (2015). Manipulation of the phenotypic appearance of individuals in groups of laying hens: effects on stress and immune-related variables. Stress, 18(6), 710-717. https://doi.org/10.3109/10253890.2015.1078306

- Liste, G., **Campderrich, I.,** de Heredia, I.B. and Estevez, I. (2015). The relevance of variations in group size and phenotypic appearance on the behaviour and movement patterns of young domestic fowl. Applied Animal Behaviour Science, 163, 144-157. http://dx.doi.org/10.1016/j.applanim.2014.11.013

- Marin, R.H., Liste, G., **Campderrich, I**. and Estevez, I. (2014). The impact of phenotypic appearance on body weight and egg production in laying hens: a group-size- and experience-dependent phenomenon. Poultry science, 93:1-13. http://dx.doi.org/ 10.3382/ps.2013-0370.

National and international conferences

- **Campderrich, I.**, Liste, G and Estevez, I. (2017). The unforeseen consequences of a "bad hair day". Annual Meeting of the Poultry Science Association, Orlando, 17-20 of July (Oral presentation).

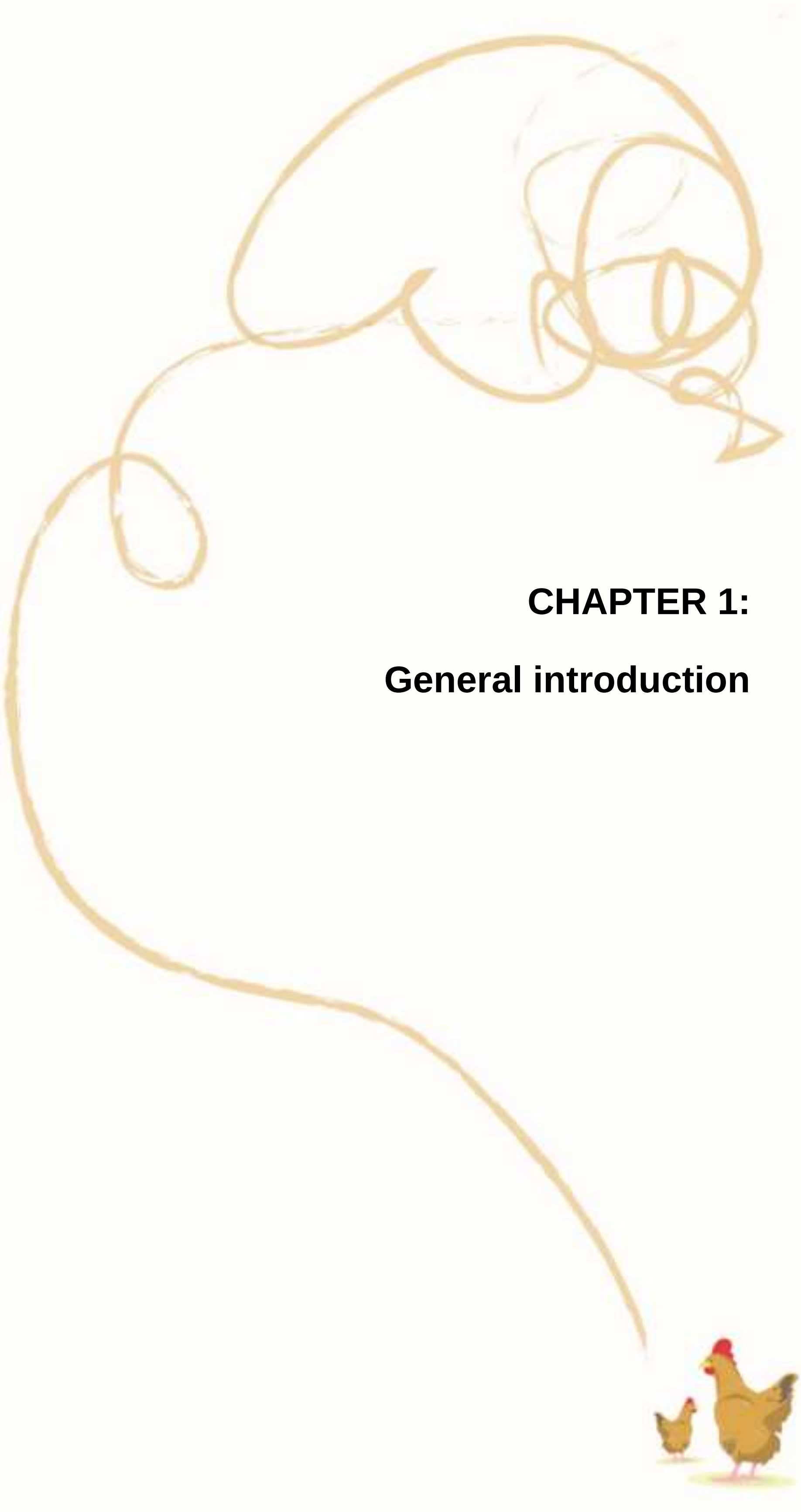
- Liste, G., **Campderrich, I.** and Estevez, I. (2016). Hens judge from appearance: changing phenotypes in stable groups of different size. In Proceedings of the 50th Congress of the International Society for Applied Ethology, 410pp. 12-15th July, Edinburgh, United Kingdom (Poster).

- **Campderrich, I.**, Liste, G. and Estevez, I. (2015). La apariencia importa; Impacto de la alteración fenotípica en gallinas de puesta. LII Congreso Científico de Avicultura, WPSA-AECA, Màlaga 28-30 de Octubre, 2015 (Poster)

- **Campderrich, I.,** Liste, G. and Estevez, I. (2014). Apariencia fenotípica en gallinas de puesta: la apariencia importa. XV Congreso Nacional y XII Iberamericano de la Sociedad Española de Etología. Barcelona del 3 al 5 de September (Oral Presentation).

- Liste, G., **Campderrich, I.** and Estevez, I. (2014). Analysis of movement in pullets: benefits of larger groups. 49º Congreso Internacional de la ISAE. Vitoria 29 de Julio-2 de Agosto (Oral Presentation).
- **Campderrich, I.**, Liste, G. and Estevez, I. (2014). Phenotypic appearance in laying hens: the looks matter. 49º Congreso Internacional de la ISAE, Vitoria 29 de Julio-2 de Agosto (Poster).
- Marin, R.H., Nazar, F.N., Liste, G., **Campderrich, I.** and Estevez, I. (2014). The impact of phenotypic appearance on stress responses in laying hens: Is it a group size dependent phenomenon? 49º Congreso Internacional de la ISAE, Vitoria 29 de Julio-2 de Agosto (Poster).
- **Campderrich, I.,** Liste, G. and Estevez, I. (2013) La relevancia de la apariencia fenotípica sobre las interacciones agresivas en gallinas durante la recría. En el 50 Congreso Científico de Avicultura, Seminario de la WPSA-AECA, Lleida 2-4 de Octubre (Poster).
- Liste, G., **Campderrich, I.** y Estevez, I. (2013) Tamaño de grupo durante la recría: aspectos comportamentales, actividad y crecimiento. En el 50 Congreso Científico de Avicultura, Seminario de la WPSA-AECA, Lleida 2-4 de Octubre (Poster).
- Marin, R.H., López, D., Liste, G., **Campderrich, I.** y Estevez, I. (2013). Relación entre el tamaño de grupo y el impacto de cambios en la apariencia fenotípica de

ponedoras sobre la postura de huevos. En el 50 Congreso Científico de Avicultura, Seminario de la WPSA-AECA, Lleida 2-4 de Octubre (Poster).



CHAPTER 1:

General introduction

1.1 The Laying hen

The domestication of chickens was initiated in Asia around 8000 years ago (West and Zhou, 1988) being the red jungle fowl (*Gallus gallus*) the most plausible ancestor for all current breeds of domestic chicken (West and Zhou, 1988, Fumihito et al., 1996). However, a possible hybridation with the grey jungle fowl (*Gallus sonneratii*) along the domestication process cannot be totally discarded (Eriksson et al., 2008). Still today, wild populations of red jungle fowl inhabit forests and semi-open areas such as field edges, groves and scrublands at the south east of Asia (including India) (Al-Nasser et al., 2007). All domestic chicken breeds used today are the result of thousands of years of selection performed by humans that during the last century was mainly focused on obtaining two main birds lines the meat and, the egg-producing line, which is the subject of this Thesis. Both chicken lines have been selected for increased productive efficiency, either in growth rate or in egg laying efficiency (Appleby et al., 2004). While the red jungle fowl produces 10 to 15 eggs in a year, laying hens produce an average of 300 eggs/year (Romanov and Weigend, 2001). Despite the long relationship with humans it was not until the last century that the poultry industry was fully developed. As a consequence of this development, the social and physical environment where chickens are maintained for production has been completely modified as compared to their natural environment, impacting on the current commercial breeds' phenotypes (Ericsson et al., 2016). In this Thesis the consequences that the modifications of the social and physical environment entail to the welfare of domestic hens will be explored.

1.2 The origins of welfare in poultry production systems

Between 1960s and 1980s poultry production systems transformed from small-scale rural enterprises onto a vital economic branch of animal production. This change implied that flock sizes increased exponentially and production systems were intensified (Frölich et al., 2012). This intensification raised public ethical concerns regarding rearing conditions of the animals used in productive systems. In particular, the publication of the book “Animal machines” by Ruth Harrison in 1964 deeply shocked the society. This book described the intensive egg production cycle literarily as a “factory farming system”. Intensive egg production has been, since one of the most criticized systems due to the restrictive conditions of its rearing environment. Such pressure determined a massive change in current production conditions in Europe as unfurnished cages are illegal and minimum space requirements have double. Ethical concerns raised by the society led to strong social movements in the UK and to the first governmental report in 1965 “The Brambell Report” (Report of the Technical Committee to Enquire into the Welfare of Animals Kept under Intensive Livestock Husbandry Systems). “The Brambell Report” stated that farm animals should have at least the freedom “to stand up, lie down, turn around, groom themselves and stretch their limbs”. Nowadays, this report is still known as the “Brambells’ Five Freedoms”. This document was later revised by the Farm Animal Welfare Council (FAWC adapted in 1993). This revised version was called the **“Five Freedoms” (Fig 1),** in recognition of the work done by the team headed by Brambell.

The updated and still considered “Five Freedoms” are presented below:

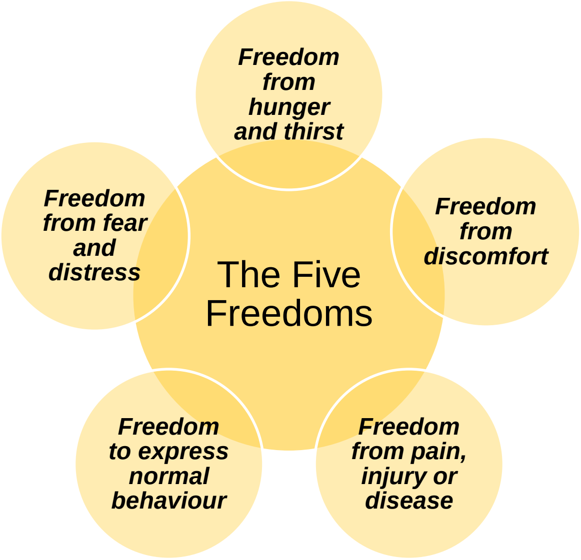


Figure 1. *The Five Freedoms in animal welfare.*

- **Freedom from hunger and thirst:** by ready access to fresh water and a diet to maintain full health and vigour.
- **Freedom from discomfort:** by providing an appropriate environment including shelter and a comfortable resting area.
- **Freedom from pain, injury or disease:** by the prevention or rapid diagnosis and treatment.

- **Freedom to express normal behaviour*:*** by providing sufficient space, proper facilities and company of the animals' own kind.
- **Freedom from fear and distress:** by ensuring conditions and treatment which avoid mental suffering.

All current European Union Legislation and animal welfare protocols are based on assuring these five freedoms to production animals.

1.3 Legislation for laying hens in the EU

Current European Directive (1999/74/EC) establishes "minimum standards for the protection of laying hens" and bans the use of conventional cages in all Member States from January 2012. This legal reform pursues the objective of better adapting production conditions to ensure minimum welfare standards for laying hens. Some of the more important actions required by law for egg production is the provision of additional space per bird. While conventional cages provided an average of 500 cm^2 per bird, furnished cage systems require minimum 750 cm^2 per bird. Space requirements for aviary systems, with or without outdoor access, are set at a maximum density of 9 birds/m^2, 6 birds/m^2 for organic production. Additionally, laying hens under current regulation must be provided with perches, nesting space, litter (that allows dust bathing, pecking and scratching) and unrestricted access to food.

Even though regulations are directed towards the improvement of birds' welfare, welfare is not warranted just by their implementation. It is well known that the provision of more space per bird is translated in an increase of behavioural opportunities (Förlich et al., 2012). This is good from an animal welfare perspective, but also increases the possibility to develop undesired behaviours such as aggression, feather pecking and cannibalism (Sossidou and Elson, 2009), which may ultimately impair welfare. Although a lot of work has been done, there are still questions to ensure birds' welfare, especially concerning their social behaviour given the large groups in which hens are normally maintained. The relevance of the early rearing conditions that laying hens encounter close after hatching is still poorly explored and may have large consequences on the birds´ behaviour and their ability to withstand challenges. A clear objective of the present work is to provide science-based knowledge on this topic which may allow us to offer tools for improving laying hens' welfare. To accomplish this objective, the first crucial step is to define the concept of welfare to be used, its evolution and how will be interpreted in the close future.

1.4 The animal welfare concept

An interesting reflection that can emerge from the Five Freedoms is that there is no simple, nor single way to define and measure animal welfare in production systems (even if we think about European countries). According to previous research animal welfare is a multidimensional concept difficult to summarize in a single sentence. Prestigious animal welfare scientists such as

Dawkins (1983) and Broom (1986) after an exhaustive literature review, proposed that welfare should include three main aspects: the animals' physical and mental health, and the animals' ability to adapt to its environment without suffering. Fraser (2004) proposed that besides assuring their biological functioning, their affective state and the possibility they have of living natural lives should be considered. Consequently, animal welfare is a complex issue and should be assessed by a combination of multilevel approaches. Welfare should not be treated as a discrete concept as it is not good or bad *"per se*". Instead, welfare varies on a continuum scale from poor to good, sometimes changing during the life of an individual (Broom, 1991). This indicates that welfare of domestic species needs to be regularly assessed at different stages along the production cycle to be truly informative. This approach would allow to correct situations that may cause welfare impairment.

An additional point to be considered is that no life can be completely free from stress or negative experiences (such as thirst, hunger, pain, discomfort, malnutrition, diseases, injuries) even in wild animals or during short periods of time (Mellor, 2016). However, a challenge to be faced during the next years is the need not only to ensure a reduction of negative experiences for farm animals, but to come up with innovative systems that allow animals to have more opportunities of experiencing positive emotions such as: comfort, pleasure, interest and a greater sense of control (Mellor, 2015; Mellor, 2016). One important point to reach this objective is to enforce the bird physical and mental ability to cope with challenges, and consequently, it is necessary to provide additional knowledge to work in this direction. This Thesis specifically investigates how laying hens' welfare can be affected by the modification of the social and physical environment. Valuable information that may help farmers, engineers and legislators to better design poultry production systems in order to prevent stress or ameliorate its consequences will be provided.

1.5 Stress in laying hens

Stress assessment and the study of its consequences in production environments is a commonly used tool to evaluate animal welfare (Broom, 2001; Morgan and Tromborg, 2007). Selye (1973) defined stress as “a nonspecific response of the body to any demand made upon it”. This definition suggests that nonspecific responses can be either caused by negative or positive experiences such as: exposure to extreme temperatures, metabolic challenges, as well as exercise, sexual excitement or pleasure. Other stress definitions also including the response to both positive and negative experiences were formulated by Chrousos and Gold (1992). They defined stress as a “state of disharmony” or a “threat to homeostasis”, definitions implying that all those stimuli able to alter the state of homeostasis should be considered stressors. Koolhaas et al., (2011) suggested in a very interesting review that stress occurs when an individual perceive a negative stimulus and the response to it exceeds the adaptive capacity in the organism. Other physiological changes due to a perceived positive event or emotion should be regarded as normal physiological mechanisms and not included in the stress concept. Lastly, the stress response has been defined as the self-constructed security system that alerts us from a potential danger (Ericsson, 2016). Therefore, when animals are exposed to an internal or external potential stressor their body responds at various levels simultaneously preparing the individual to act against the threat (Ericsson, 2016). Problems arise when the stress response is too strong, or persists for a long period of time, and the individual becomes no longer able to cope with it in an effective way. When this happens, stress can have negative effects for the individual, provoking a cascade of negative consequences, mainly produced by a redistribution of resources leading to impairment of the biological functions involved (Moberg, 2000). Biologically, the stress response involves changes in behavioural, metabolic, immunological and neuroendocrine mechanisms (Warriss, 2010).

Two main types of stress responses have been described: the acute short-term defined as the *fight or flight* response originally described by Cannon (1929), and the long term or chronic response originally described by Selye (1946). In the acute (short term) response behavioural and physiological systems are activated preparing the animal for a rapid reaction to any environmental change, whereas the chronic (long term) response involves an important adjustment of the autonomic and neuroendocrine systems (Barnett and Hemsworth, 1990). After an acute stressor, the sympathetic nervous system is activated leading to hormonal responses with catecholamines release to the bloodstream (adrenaline and noradrenaline) (Romero and Butler, 2007). This type of response results in heart rate and blood pressure raise. Resources are allocated to the muscles, thus preparing the individual for fighting or flying. At this moment, the animal can avoid further responses behaviourally moving away from the original stressor (Moberg and Mench, 2000). A second hormonal response due to the activation of the HPA axis culminates with the release of glucocorticoids (in particular corticosterone in the case birds). Sympathetic nervous system activation tends to be quick and short while the release of glucocorticoids requires more time and has long lasting effects. When high levels of glucocorticoids are sustained for a long period, defining a scenario known as chronic stress, biological systems may result deeply impaired. Reported effects of chronic stress are: an impaired immune function (O'Mahony et al., 2009), deteriorated growth (Satterlee et al., 2000), increased aggression rates (Veenit et al., 2013) and behavioural abnormalities (Jones et al., 2010). The relevance of stress consequences for an individual depends on the duration, intensity, type of stressor and pervious experience, as well as, the animal state at the moment of the stress exposition. Additionally, factors such as the perceived controllability and predictability are essential in modulating the impact of the stress response (reviewed in Koolhaas et al., 2011). Knowing the effects, the stressor that commonly affect production birds has and monitoring their state regularly will give us better tools when it comes to managing the groups and designing safer production environments.

1.6 Social and environmental challenges to the welfare of laying hens

Birds used in poultry production systems are commonly exposed to multiple factors capable of inducing stress (Appleby et al., 1992; Fraser and Broom, 1997; Lara and Rostagno, 2013). When animals live in captivity the social and physical environment differs greatly from the environment where their ancestors evolved. Farm layout and animal group arrangements for productive lines have been designed by breeders and farmers, most of the time considering production aspects but without taking in consideration specific social and environmental needs. In this Thesis the effects of exposing laying hens to different social and environmental challenges will be explored together with the associated welfare consequences.

1.6.1 Social challenges

1.6.1.1 Differences between groups in the wild and in productive systems

Groups of wild fowl are normally a harem composed of a dominant male, the alpha male, a few subordinate males and several females of different ages (McBride et al., 1969; Collias and Collias, 1996). Group structure is known to vary across seasons: during breeding females leave the male to incubate and raise their broods alone while groups of only males are form (Collias and Collias, 1967; Sullivan, 1991). In the wild, fowl are able to discriminate between group members (Bradshaw, 1991) and use this ability to build and maintain their social hierarchy

(Abeyesinghe et al., 2009). Nevertheless, when it comes to poultry environments, things are different from those observed in the wild. It may be difficult to find out which is the optimum group size and composition for productive purposes (see Estevez et al., 2007 for an extended review). In productive systems, eggs are taken away from the hens after lay and are artificially incubated. New-borns chicks do not have contact with their mothers and are normally kept on aviaries with a large number of conspecifics (Rodenburg et al., 2008). Few hours after birth, pullets are sent to the rearing facility (Widowski and Torrey, 2018) until reaching maturity at 16-17 weeks of age (Leinonen et al., 2012). As adults, laying hens are transferred to production facilities and kept in large groups that vary in size depending on the production system (enriched cages or aviaries with or without access to outdoor space, (Appleby, 2003)). As mentioned, group composition and size may vary depending on the production system and birds' age. Nevertheless, laying hens will most likely be kept in much larger groups than those observed in wild.

Groups' composition is also different from that observed in natural groups (Keeling and Gonyou, 2001; Eklund and Jensen, 2011); single-sexed groups of the same age and homogeneous phenotypes due to genetic selection. However, it is easy to think that farm animals would likely deal better with a social environment, referring to group size and social structure, that resembles their natural group composition. Social behaviour, communication and recognition abilities of individuals evolved to deal with their ancestral live conditions (Keeling and Gonyou, 2001). In fact, studies such as the one performed by Manteca

(2015) in zoos, may support this hypothesis as they postulate that animals living in captivity may suffer from social stress when: 1) they are kept in groups which size and composition is different from that of the groups observed in the wild, 2) they are mixed with other conspecifics with which they had no previous contact or 3) it is not possible for all the animals to have access to resources at the same time. It should be considered whether the rearing environment provided to the domestic fowl is the best for assuring welfare, without underestimating the species' clearly demonstrated plasticity and adaptive capacity (Roll et al., 2008). As this may be a complex issue, the challenge in the first part of this Thesis (chapters II and III) will be acquiring the knowledge referred to how laying hens' social interaction may be influenced by intrinsic characteristics of the group (homogeneity or heterogeneity of the group) and its size (in a range of group sizes close to the numbers observed in the wild).

1.6.1.2 How do laying hens deal with sociality?

Living in a social group involves advantages but also has associated costs (see Estevez et al., 2007 for an extended review). Aggressive contests are an essential part of animals' life as they serve to establish hierarchies and solve competence for resources such as mates, food or territory (Queiroz and Cromberg, 2006). Being involved in aggression implies some costs depending on the intensity, frequency and duration of the aggressive interactions. The most common forms of aggression described for laying hens are:1) those that may involve physical contact such as aggressive pecks, leaps, or chases (active acts and energetically expensive) or 2) those that don’t involve physical contact: like displacements or threats (passive acts that are cheaper in energetic terms) (see Estevez et al., 2002 for a detailed description of the behaviours mentioned). Intensity, duration and frequency of aggressive acts will determine its effects. In some cases, pullets and laying hens can be bruised or injured, experience negative emotions, or even suffer from stress as a consequence of high intensity aggressive interactions (Bilcík and Keeling, 1999; D’Eath and Keeling, 2003; Rodenburg, and Koene, 2007; Buitenhuis et al., 2009). When this occurs, welfare is challenged and productive indicators may be affected, which can even lead to important economic loses. It can be argued that to ensure good welfare and to maintain production rates, being able to monitor aggression in productive systems is important. Deep knowledge referred to these issues may help finding ways to reduce its occurrence, act when is needed or alleviate its consequences.

Conversely, affiliative behaviours seem to have a series of pleasant effects on the animals inducing the release of oxytocin (Neumann, 2008), a hormone that contributes to the reduction of the stress response (Amico et al., 2004; Neumann et al., 2000). Among the positive effects attributed to affiliative behaviours such as allopreening, the reduction of aggression levels and the facilitation on the formation and maintenance of social bonds are the most relevant (Harrison, 1965; Wood-Gush and Rowland, 1973). Affiliative behaviours are considered positive indicators of welfare (Boissy et al., 2007). According to this, it is important to design production systems in such a way that promotes a positive balance between affiliative and aggressive acts, thus ensuring better welfare. Different ways of providing a positive balance for laying hens could be by exploring which circumstances favour the expression of affiliative behaviours, or to investigate how to reduce aggression or the factors that can induce aggression in the productive environments. The balance between the frequency and intensity of these two social contexts (affiliative vs aggressive) may be regulated by factors such as the degree of phenotypic diversity among group members or the size of the groups, as explain in the following section. Such factors are the main focus of study in the first chapters of this Thesis.

1.6.1.3 The relevance of phenotypic appearance on social interactions in laying hens

Different studies suggest that social interactions in laying hens do not occur randomly among group members. Instead, it seems there are individuals which present certain characteristics that make them more vulnerable to become targets of aggressive interactions. The existing literature describes targeted physical features such as a low body mass, a small comb size or a manipulated plumage colouration (Cloutier and Newberry, 2002; Estevez et al., 2003; Dennis et al., 2008). Those characters make individuals singular or distinguishable within their group. These traits could vary along ontogeny, due to individual differences in feeding, growing rates or behaviour which may suggest that in certain moments those individuals may be more vulnerable to attacks by conspecifics. On the other hand, it has been described that shared physical traits or characters, or in other words similarity between conspecifics, is related with a stronger tendency to cooperate and interact in a less aggressive manner (Hamilton, 1964 a, b; Lizé et al., 2006) showing a trend to establish greater social cohesion. Mechanisms such as kin selection (Hamilton, 1964 a, b) or phenotype matching (Grafen, 1990), developed early after hatching, may settle the basis to explain such behaviours.

Imprinting is a process by which domestic fowl (and other species) orient themselves just after hatching and learn to follow the first biologically relevant social model they encounter (Lorenz, 1935; Hess, 1958). This is a crucial process because it increases the chances of survival and facilitates complex social learning and development (Di Giorgio et al., 2017). Also, relevant for the present Thesis, imprinting helps chicks to stablish the physical traits that allow them to recognize mates. Phenotypic appearance (PA) is, at this level, a key source of information. PA contains information that helps identifying kinship based on genetic relatedness among different individuals (Hamilton, 1964 a, b). Alternatively, a simpler mechanism such as phenotype matching would allow group member and species recognition by a system based on learning the phenotype of familiar relatives (Grafen, 1990; Barnard, 1991) during early development. According to phenotype matching, animals would be able to develop a phenotypic template that they will later use in any social context to compare towards the phenotypes of others and distinguish them as familiar or unfamiliar individuals (Mateo and Johnston, 2000; Gerlach and Lysiak, 2006). This mechanism may also help detecting unhealthy individuals that may expose them to new pathogens (Zuk et al.,1990; Hughes and Cremer, 2007) or even identify unrelated “invaders”, individuals that may outcompete locals for valuable resources. Once recognized, they may aggressively exclude or isolate those “dangerous”/” strange”/ “unfamiliar” individuals from the group.

The impact of PA over social interactions can be determined by the size of the group, the frequency of the altered phenotype and the time when the alteration takes place. Consequently, it seems relevant to include those aspects when exploring its effects. Group size determines how laying hens interact with each other, build their social system and recognize others. When kept in small groups the social behaviour of domestic fowl is similar to that of their wild ancestors; they stablish a dominance hierarchy or ‘pecking order’ that is determined by aggressive interactions (Collias et al., 1966). One of the requirements to form and maintain a hierarchical system is that animals recognize each other (McBride and Foenander, 1962) and remember the outcome of their aggressive encounters (Cloutier et al., 1995). However, when individual recognition of all group members is complicated or not even possible because the group is too large, birds may switch to dominance relationships determined by physical features such as body and comb size that represent badges of status (Pagel and Dawkins, 1997). Is in those large groups then were the impact of altering the PA of the birds theoretically would have larger consequences.

On the other hand, the proportion of animals altered within a group can also be a relevant issue to consider. Laying hens may form their phenotypic templates by imprinting early after hatching based on the existing phenotypes in their close social environment (Bateson, 1979; Bateson, 1990; Gerlach and Lysiak, 2006). When more than one phenotype coexists in a group, it would be expected that birds will tend to identify themselves with the most common one or with the diversity of existing phenotypes. This may indicate that the frequency of different phenotypes within a group would also have a relevant role in determining social interactions. In fact, frequency dependent effects have been found on social interactions in previous studies (Dennis et al., 2008). Dennis et al. (2008) found that when the manipulation of the PA of different bird proportions in a group (20, 50 and 100%) were applied, aggressive interactions were affected. Birds with altered appearance were the main receptors of the aggression, especially when only 20% of the birds in the group were altered but not when the proportion of altered birds increased. The altered birds in the 20% manipulated group presented a lower body mass and altered levels of stress hormones when compared to their unaltered pen mates, which ultimately led to a poorer welfare status.

Another relevant issue would be the temporal occurrence of phenotypic diversity. Specifically, diversity in PA occurring early in life would be easy to incorporate into the group social dynamics. Early in life, birds seem to be more plastic to learn and modulate/configure their templates according to the phenotypes they find in their close proximity (Marin et al., 2014). Contrarily, during adulthood, when the templates are already well-established, the emergence of new phenotypes would be expected to be much more disruptive to the population. To the authors knowledge this is a question (explored at chapters 2 and 3) that has not been tested before in laying hens.

1.6.2. Environmental challenges affecting laying hens' welfare

Birds in productive systems may be exposed to multiple challenges far from the ones they would deal normally in the natural environment. They may be expose to artificial light, unfamiliar and sudden sounds, odours, temperature alterations, confinement, lack of retreat space, restricted feeding and foraging, forced human proximity, and the exposition to more challenging social groups (Campo and Dávila, 2002; Mashaly et al., 2004; Morgan and Tromborg, 2007; Lay et al., 2011; de Haas et al., 2013). The exposition to different challenges, alone or combined, may lead to a variety of consequences for the birds that are still being studied. There exists no legislation on the handling and management of newly hatched chicks or younger pullets besides the general statement that mentions that animals should be treated well. A deeper understanding of the impact that these early situations have on the birds' behaviour and physiology is important from the animal welfare perspective.

Studies on the effects of early life challenges may contribute to understand the evolution of phenotypic diversity in the populations (Göerlich et al., 2012). Adaptive plasticity theory suggests that the inputs that animals receive along their lives can produce long lasting alterations in the individuals. These alterations can, in turn, transform the phenotype providing each individual with the capacity of better dealing with the challenge, or, on the contrary result on a less adapted phenotype (Nettle and Bateson, 2015) which may carry fitness costs. In the second part of this Thesis we will explore the effects of exposing laying hens to different and consecutive environmental challenges. Birds in this work were exposed to an acute period of cold stress (Cold Stress Treatment (CSTR)) early after hatching. As CSTR may affect different aspects of birds' welfare, the study included and evaluated the response of the pullets at the morphological, immunological and behavioural level. Understanding the impacts that such an early challenge can have on the birds' global welfare status can help us to develop better management practices for this crucial phase during development. Additionally, whether the stress experienced early in life can affect birds' later responses against the new challenges (by exposing them to an Intermittent Stressful Challenges Protocol (ICSP)) was also explored. The reason for exposing birds to this chain of challenges and check for their effects is to resemble a series of problems associated to actual rearing conditions that could potentially overlap. In these experiments the effects and duration of unpredictability and uncontrollability were explored.

1.7 Introduction of environmental complexity as a tool to counteract negative effects of challenges

Wild red jungle fowl live most of their live in complex three-dimensional spaces such as forests and semi open areas with good amount of vegetation (Collias and Collias, 1967), which serve as protection from ground and aerial predators or to avoid being detected by conspecifics (Elton, 1939; Stahl et al., 2002). Instead, domestic hens are kept in barren bidimensional environments, at least until they reach adulthood and features such as perches, nest space and litter are offered to meet UE legislative requirements. The question then is whether the space offered in production conditions is behaviourally relevant for the development of the basic behavioural patterns (Price 1999) or, if it serves to improve their biological functioning (Newberry, 1995). Considering this, the structural characteristics of the space provided should allow domestic fowl to satisfy their need of locomotion and activity according to their age, be comfortable and well-designed for resting and recovering, allowing them to properly respond to potential stressors. The typical anti-predator behaviour for wild red jungle and domestic fowl is based on remaining still in the vegetation, grouping together close to protective areas or vegetation, even when provided artificially (Newberry and Hall, 1990; Newberry and Shackleton, 1997; Cornetto and Estevez, 2001; Rodriguez-Aurrekoetxea et al., 2014) or, jumping up to branches of trees or perches (Newberry et al., 2001). Among the positive effects from perches and cover, reduction in the incidence of feather pecking (Huber-Eicher and Audige, 1999), improvement of exercise and leg condition (Haye and Simons, 1978; Newman and Leeson, 1998; Bizeray et al., 2002; Leyendecker et al., 2005; Ventura et al., 2010), decrease on aggression and disturbances (Ventura et al., 2012), and a better use of space in and out poultry facilities (Leone et al., 2007; Rodriguez-Aurrekoetxea et al., 2014) are some of the benefits reported in the literature. Apart from the mentioned benefits, it has been suggested that giving prey species the opportunity to take cover or jump onto perches and platforms may be considered a form to control their environment (Keeling, 1997). Such perceived controllability helps animals to better cope with stress challenges (Koolhaas et al., 2011) and is, at the same time, associated with a positive emotional state (Boissy et al., 2007) of safety or security. The negative effects that the lack of a three-dimensional space during early developmental stages have on the welfare of pullets has been poorly explored. However, it has been reported that the lack of perches at early ages may affect the proper development of spatial skills (Gunnarson et al., 2000). Consequently, it was proposed that providing an early enriched environment will contribute to improve welfare, increase the possibilities to express natural behavioural responses against stress, and consequently, mitigate the effects of the challenges faced along production cycle. Some of these questions will be experimentally explored on Chapter 4.

Finally, Chapter 5, will be devoted to jointly discuss the results obtained during the development of this work (Chapters 2, 3, 4). Based on the knowledge acquired, different options to improve the hens' conditions along the production cycle will be proposed. This Thesis ends with a conclusion section based on the results obtained.

1.8 Hypothesis and objectives

The general aim of this Thesis was to investigate the consequences of exposing laying hens to different social and environmental challenges. The first part of this work covered the social aspects and was devoted to explore the effects of the alteration of phenotypic appearance on social dynamics at different ontogenic stages of a laying strain of domestic fowl reared at different group sizes (GS). The second part was dedicated to investigate the possible summative or cumulative effects from diverse early environmental stressors that commonly affect poultry under commercial conditions, and test whether those effects can be ameliorated by the provision of a complex environment (CENV).

The specific aims and predictions for chapters 2-4 are:

Chapter 2: To determine the combined effects of different degrees of variability in the phenotypic appearance (PA) of the populations with variations in GS, on the development of affiliative and aggressive social interactions in young laying hens. It was hypothesized that altering the degree of diversity in a group by altering their PA would determine the frequency and direction of the aggressive and affiliative interactions performed for young laying hens. Such effects would be GS and PA frequency dependent. We predicted that:

a. Birds presenting the less common phenotypes would be at higher risk of being targeted in aggressive encounters, while they will show lower levels of affiliative interactions.

b. The effect of PA would be more remarkable in groups with the lower proportion of individuals altered and in smaller GS as the same proportion of altered birds involves less birds

Chapter 3: To study the effects of sequentially altering the PA of different proportions of individuals within originally homogeneous adult groups of laying hens, with variations in GS on the development of aggressive social interactions. It was hypothesized that the manipulation of the PA in socially stable groups of adult birds will produce a sudden escalation on aggressive interactions. This escalation may differ in intensity depending on the GS and the frequency of newly altered individuals in the group. We predicted that:

> a. Aggression will increase in those groups altered during adulthood and compared to controls. b. Aggression will be specifically directed towards recently altered birds.
>
> c. The response to the alteration of the phenotype will be stronger the lower the proportion of altered birds.
>
> d. The impact of the PA manipulation will be smaller in small groups where individual recognition is assumed (e.g. 10 birds) as compared to larger groups where individual recognition is less likely**.**

Chapter 4: Determine if the early provision of a complex environment (CENV) can ameliorate the effects of early stressors or buffer against future unpredictable environmental challenges in young laying hens. Birds’ stress response was assessed at the immunological, morphological and behavioural level. It was hypothesised that providing birds with a CENV during early life, will better prepared them to cope with those stressful events. We predicted that:

- Experiencing a CENV would attenuate the impact of acute stress and improve birds' ability to cope with future unpredictable challenges.

CHAPTER 2.

Group size and phenotypic appearance: Their role on the social dynamics in pullets.

2.1 Abstract

Non-caged production systems offer greater freedom of movement and behavioural opportunities to pullets, which may also include the occurrence of undesired behaviours. The incidence of such behaviours may be affected by group size but also by the group members´ phenotype. This study was designed to explore the effects of group size and phenotypic appearance in the social dynamics of pullets. A total of 1050 day old Hy-line Brown was randomly assigned to 45 pens at 3 group sizes (GS): 10, 20 and 40 birds (constant density 8 hens/m^2). For all GS treatments, the phenotypic appearance (PA) of different bird proportions was modified with a black mark at the back of their head. Two types of populations were studied: homogenous (0, 100%) with all pen members presenting the same marked (M) or unmarked (U) phenotype, and heterogeneous (30, 50, and 70%) were the two phenotypes (M and U) coexisted in the same pen. All pens were observed during rearing on alternate weeks. Aggressive and affiliative social interactions performed among group members were registered together with their PA (M or U). The observed and expected mean frequencies for all phenotype combinations involved in the social interaction were calculated: MM, MU, UU and UM, where the givers´ phenotype is indicated by the first letter and the receivers´ phenotype by the second letter. Data were analysed with mixed model ANOVAs that included GS and PA as fixed factors for the general model, and GS, PA and type of interaction (MM, MU, UU, UM) to determine the direction of the interaction in the case of heterogeneous

groups. Both aggressive (threats) and affiliative interactions (exploratory pecking and beak pecking) were more frequent in small (10) than in large (20, 40) GS (P < 0.05) regardless of PA (PA, P > 0.05; GS × PA, P > 0.05). Aggressive interactions, however, occurred at a higher than expected frequency in heterogeneous GS 20, and were predominantly from U towards M birds (UM; P < 0.05), as compared to MU or UU. The significant interaction among GS × PA × Type of interaction for affiliative behaviours did not show a clear relationship. Overall, the results obtained showed that both aggressive and affiliative interactions occurred at a higher frequency in the smallest GS 10. Although the effects of PA were not as clear as expected, these results provide evidence of the directionality of the aggressive interactions from U towards M birds especially at intermediate GS 20.

2.2 Introduction

Non-cage production systems offer greater freedom of movements and behavioural opportunities to laying hens, thus are considered beneficial from a welfare stand point (Frölich et al., 2012). Some studies however, suggest that increasing freedom of movement may also have negative consequences for the welfare of laying hens by increasing the occurrence of undesirable behaviours such as aggression, feather pecking, and cannibalism (Sossidou and Elson, 2009), which can easily spread in the population (Cloutier et al., 2002). Even though the consequences of undesired behaviours may not be as dramatic for pullets as in adult laying hens it is important to prevent their appearance during rearing.

In addition to the greater freedom of movements, the larger group sizes (GS) generally used in non-cage systems may lead to increased social instability and aggression. When domestic fowl are kept in small groups their social behaviour appears to be similar to that of their wild ancestors, the red jungle fowl (*Gallus gallus*), establishing a dominance hierarchy that is determined by aggressive interactions (Collias et al., 1966). However, it has been suggested that in large GS the social hierarchy is replaced by a tolerant system characterized by low aggression (Estevez et al., 1997; Estevez et al., 2003). Pagel and Dawkins (1997) proposed that the social strategy of a group will switch when the costs of establishing and maintaining a dominance relationship outweigh the benefits. It has been suggested that this breakdown may occur at intermediate GS of around 30 individuals (Keeling et al., 2003).

Several studies suggested that phenotypic appearance (PA) may also play an important role regulating social dynamics by altering the way that individuals are perceived by their conspecifics. For instance, it has been suggested that aggressive interactions do not occur randomly within flocks of domestic fowl and that some individuals are at higher risk of being involved in aggressive encounters. It has been observed that aggression is mainly directed towards subordinates (Mc Bride, 1960; Guhl, 1968; Queiroz and Cromberg, 2006) or individuals with particular phenotypic characteristics such as low body mass, small comb size or manipulated plumage coloration (Cloutier and Newberry, 2002; Estevez et al., 2003; Dennis et al., 2008). Keeling et al. (2004) also indicated that the expression of a wild recessive allele at PMEL17 gene that controls plumage melanisation, may predispose chickens to become victims of feather pecking. Moreover, pigmented birds in their study (within a white hen population) were found to be more vulnerable to feather-pecking when they were relatively common (22–26% of the birds showing the pigmented phenotype). Frequency dependent effects were also found when manipulation of the phenotypic appearance of different bird proportions in a group (20, 50 and 100%) were applied (Dennis et al., 2008). In this study, birds with altered phenotypic appearance were the main receptors of aggressive interactions, especially when only 20% of the birds in the group were altered. The altered birds also showed lower body mass and changes in levels of stress hormones as compared to their unaltered pen mates.

It has been suggested that animals with similar PA tend to cooperate and interact in a less aggressive manner (Hamilton, 1964 a, b; Lizé et al., 2006). In terms of kin selection theory, it has been suggested that phenotype matching may be one of the mechanisms used by animals to discriminate between kin and non-kin (Komdeur and Hatchwell, 1999). Phenotype recognition would be expected to develop during the first few weeks of age while birds interact with each other. Thus, it is expected that PA treatment would affect to their aggressive and affiliative interactions during rearing.

Aggressive interactions and it effects in social dynamics in the domestic fowl have been studied extensively and are well defined (Estevez et al., 2002, 2003). Exploratory pecking and beak pecking may also have an important role in social bonding; thus, some basal levels of feather pecking have been interpreted as a form of allopreening in young birds (Bolkhuis, 1986; Vestergaard et al., 1993). Allopreening have an important role in reducing aggression levels and in the formation and maintenance of social bonds (Harrison, 1965; Wood-Gush and Rowland, 1973), and have a positive effect on decreasing stress levels in social species (Dunbar, 1991; Lewis et al., 2007).

To the authors´ knowledge no previous studies considered the effects of PA over both aggressive and affiliative interactions and no studies have been conducted in a layer strain of the domestic fowl. The aim of the present study was to investigate the effects of GS and PA on the development of affiliative and aggressive social interactions in young laying hens. We predicted that birds in larger GS will show lower levels of aggressive interactions. Birds presenting the less common phenotypes would be at higher risk of being involved in aggressive encounters while will show lower levels of affiliative interactions. The effect of PA is predicted to be less remarkable as the percentage of individuals altered increase, and in larger GS as the same proportion of altered birds involves more birds, therefore the phenotype may be encounter at a high enough frequency.

2.3 Material and methods

2.3.1 Experimental facilities

This study was performed at the experimental poultry facilities of Neiker-Tecnalia (Vitoria-Gasteiz, Spain). A large room (40 × 8 m) provided with two lines of automatic drinkers and feeders and a computerized system for light, ventilation and temperature control was used. The room was divided in 45 pens of 1.25 m^2, 2.5 m^2 and 5 m^2 (15 pens for each size) that housed groups of 10, 20 and 40 birds, respectively, at a constant density of 8 birds/m^2. The pens were built with PVC piping and plastic netting. An opaque plastic covered the lower part of the walls between pens to avoid visual contact across experimental groups. The pen floors were covered with 1.5 kg/m^2 of wood shavings. The chicks had *ad libitum* access to food and water (4 cm round feeder space/bird and 1 nipple drinker/5 birds) and were fed a commercial diet accordingly to the rearing phase.

2.3.2 Animals and rearing conditions

A total of 1150 1-day-old female Hy-line Brown laser beak trimmed chicks were obtained from a commercial hatchery (Avigán Terralta Tarragona, Spain). Upon arrival to the experimental facilities each bird was randomly assigned to a GS and PA treatment. We tested three experimental GS, 10, 20 and 40 birds per group, combined with five PA proportions of 0, 30, 50, 70 and 100% of the birds with altered phenotype in each pen. The different proportions of PA alterations were achieved by placing a black mark with a non-toxic dye on the back of the head in the corresponding number of birds in each pen (Dennis et al., 2008). All marks applied were as similar as possible, and chicks were remarked as needed according to feather growth. By this procedure we obtained: homogenous populations (0, 100%) with all members of the group presenting the same PA, unmarked (U) or marked (M), and heterogeneous populations (30, 50, and 70%) where the two phenotypes (M and U) coexisted in the same pen.

At 10 days of age all birds were marked with individual identification tags made of laminated white paper squares (3.5 × 3.5 cm). One of the numbers corresponded to the bird ID and the second to the pen number. Tags were affixed at the membrane of the wings with plastic filaments injected under the skin following the procedure described in Cornetto and Estevez (2001a). Birds were retagged at 8 weeks of age in both sides of the neck with larger tags (5 × 5 cm) due to increased body weight and growing of feathers. Every bird in the pen (M or U) had identical wing tags, so the phenotype of all individuals was the same in regard to all birds carrying tags. In addition, previous investigations showed that pecking at the tags decreased to negligible levels after the first week as birds were habituated to them in all treatments (Dennis et al., 2008).

2.3.3 Observational methods

Observations were conducted on weeks 3, 5, 7, 9 and 13 of age, between 9:00 and 14:00h. Pens were randomly allocated to an observation slot every week of observation, with around 9 pens being observed per day (45 pens per week). Each pen was observed continuously during 15 min. During this time all observed aggressive and affiliative interactions performed across group members were recorded as events, and the ID of the giver and the receiver of the interaction noted together with their PA. The definitions of the behaviours collected in the study were adapted from previous studies (Estevez et al., 2002; Riedstra and Groothuis, 2002; Newberry et al., 2007), and are presented on Table 2.1. Threats, pecks, leaps and chases were defined by Estevez et al. (2002) as aggressive interactions considered for us as negative behaviours as they are considered aggressive and can involve a cost and might trigger fear responses and injuries. All data collection was performed “in situ” by the same person, with observations starting after a five-minute habituation period to the presence of the observer. Data were collected using the software The Observer 10.0 (Noldus, Netherlands).

Table 2.1: *Definitions used for the behavioural data collection*

Behaviour	Description	Adapted from
Aggressive peck	One bird raising her head and vigorously stabbing her beak at the other bird (usually directed towards the head).	Estevez et al., 2002
Leap	A bird jumping and kicking her feet forwards at her opponent.	
Chase	One bird at the pen running after another bird for more than three steps in an aggressive manner.	
Threat	One bird standing with her head clearly raised (sometimes accompanied by raising of the neck feathers) in front of a second bird who held her head at a lower level.	
Exploratory gentle feather pecking	Gentle beak contact with the feathers of another bird without visibly altering the position of the feathers.	Newberry et al., 2007
Beak pecking	One bird directing gentle beak pecks to the recipient´s beak.	Riedstra and Groothuis., 2002

2.3.4. Statistical analyses

2.3.4.1 General treatment effects (PA: 0, 30, 50, 70 and 100% altered birds)

The mean frequency of occurrence per 15 min observation was calculated for each behaviour and pen, and then standardized according to GS. The mean frequency for the entire observation period (3–13 weeks of age) was then calculated and used as our response variable. Linear mixed models (PROC MIXED) were used for data analyses, in which GS, PA treatment and their interaction were included as fixed factors and pen as random effect. Due to the low frequencies of aggressive pecks, leaps and chases data were pooled in a single category named “Sum of pecks leaps and chases”.

2.3.4.2 Directionality of social interactions in heterogeneous groups (30, 50 and 70%)

In heterogeneous groups (30, 50 and 70%) where both phenotypes altered (M) and unaltered (U) coexisted in the same pen, we analysed the directionality of their social interactions. For this purpose, we calculated the frequency of each type of social interaction between each possible pair of givers and receivers (MM, MU, UM, UU) within each corresponding group size. The expected value (assuming that interactions occurred randomly) was also calculated according to GS and the number of M or U birds for each corresponding PA treatment. To determine the directionality of the social interactions, the differences between observed and expected values for each potential type of interaction were calculated per observation and pen and then averaged for the study period and used as our response variable. Linear mixed models (PROC MIXED) were also used here, but GS included only heterogeneous groups (30, 50 and 70%). PA treatment and the type of interaction (MM, MU, UM, UU) were included as fixed factors together with their interactions. Pen was considered as random effect.

Data were normally distributed except for the sum of aggressive pecks, leaps and chases that had to be square root transformed in order to meet the assumptions of normality. All analyses were performed using SAS 9.3 (SAS Institute, Cary, NC, USA). Bonferroni post-hoc comparisons were employed in all cases to evaluate significant differences between groups.

2.3.5 Ethical note

Animals used in this project were treated according to the legislation regarding the use of animals for experimental and other scientific purposes (Real Decreto 1201/2005). The protocol for this experiment was evaluated and approved by the Ethical Committee at Neiker-Tecnalia and the Committee of the Livestock Services at the Regional Government (Diputación Foral de Alava). This study was part of a larger project that evaluated different aspects of PA and GS on welfare, health and performance of laying hens.

2.4 Results

2.4.1 General treatment effects (PA: 0, 30, 50, 70 and 100%)

The results showed a significant effect of GS in the frequency of threats ($F_{2,30} = 8.16$, $P < 0.002$; Fig. 2.1), with groups of 10 birds showing a higher frequency of threats as compared to GS 20 and 40 (Bonferroni, $P < 0.05$). No effects of PA treatments or the interaction GS by PA treatment ($P > 0.05$) were detected for threats. Additionally, when considering the sum of aggressive pecks, leaps and chases, no effect of GS, PA treatment or of their interaction were detected ($P > 0.05$).

Regarding the affiliative behaviours, GS had an effect on the frequency of exploratory pecking ($F_{2,30} = 20.12$, $P < 0.0001$) and beak pecking ($F_{2,30} = 28.79$, $P < 0.0001$), with lower frequencies observed in larger GS (Fig. 2.2). No other effects detected.

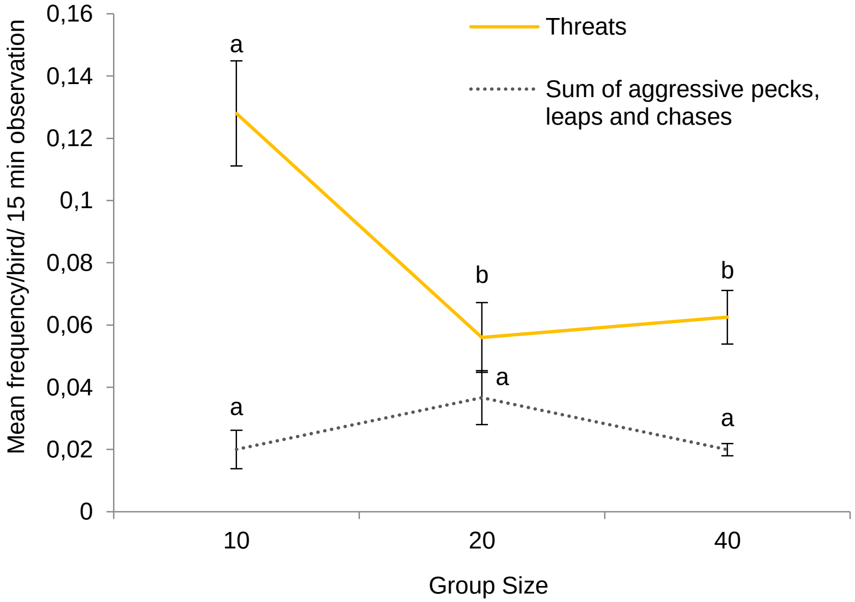


Figure 2.1 *Mean (±SE) frequency of threats and sum of aggressive pecks, leaps and chases per bird and observation across group sizes 10, 20 and 40. Lowercase letters indicate significant differences among different group sizes (P < 0.05).*

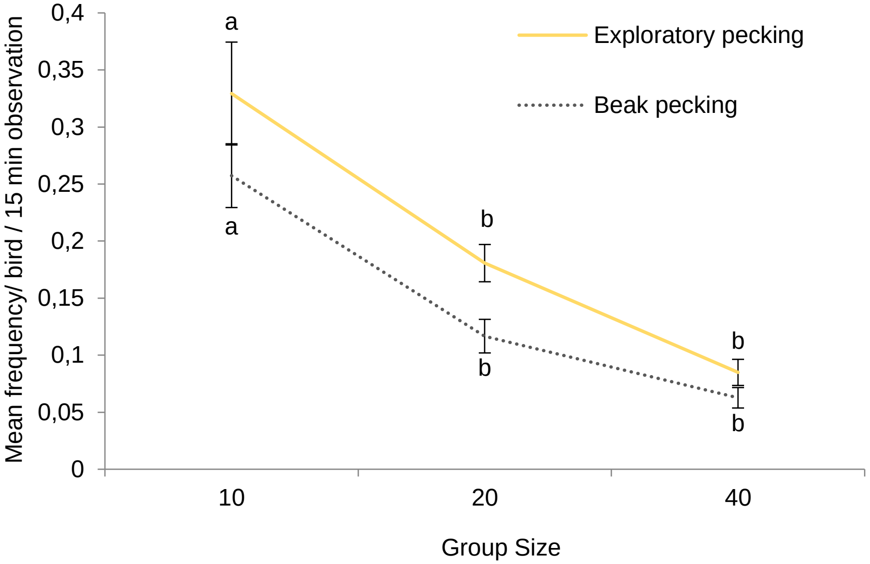


Figure 2.2 *Mean (±SE) frequency of exploratory pecking and beak pecking per bird and observation across group sizes 10, 20 and 40. Lowercase letters indicate significant differences across different group sizes (P < 0.05).*

2.4.2 Directionality of social interactions in heterogeneous groups (30, 50 and 70%)

The analyses of the differences between observed and expected values for threats in heterogeneous groups showed no variations for any of the factors included in the model ($P > 0.05$). However, the sum of aggressive pecks, leaps and chases showed a tendency for the effect of type of interaction ($F_{3,72} = 2.43$, $P = 0.07$; MM −0.015 ± 0.05, MU −0.07 ± 0.05, UM 0.12 ± 0.05, UU −0.03 ± 0.05, mean ± SE of the observed minus expected). A significant effect of the interaction of GS by type of interaction was also detected for this behaviour ($F_{6,72} = 2.55$, $P < 0.05$; Fig. 2.3). Significant differences were observed in GS 20 between the observed and the expected values for the sum of aggressive pecks, leaps and chases which were significantly higher for UM pairs (from unaltered towards altered) as compared to MU and UU ($P < 0.05$).

For beak pecking, a significant effect of GS by type of interaction was detected ($F_{6,72} = 2.45$, $P < 0.05$; Fig. 2.4), although Bonferroni comparisons were not significant ($P > 0.05$). Additionally, for the directionality of affiliative events in heterogeneous groups, we found a triple interaction among GS, PA treatment and type of interaction for both beak pecking ($F_{12,72} = 2.94$, $P < 0.01$; Fig. 2.5) and exploratory pecking ($F_{12,72} = 2.21$, $P < 0.05$; Fig. 2.6). Despite of these significant effects, the Bonferroni comparison yielded no significant differences for beak pecking or exploratory pecking ($P > 0.05$). No other effects were detected.

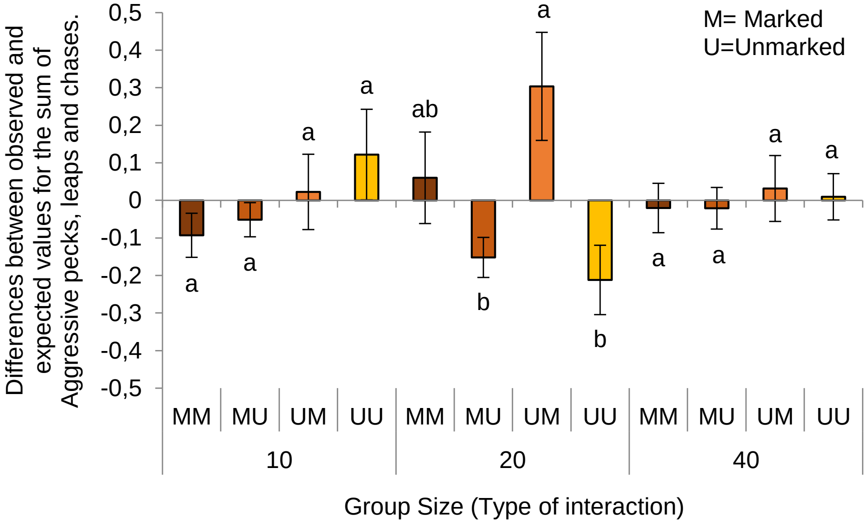


Figure 2.3 *Differences between observed and expected values for the sum of aggressive pecks, leaps and chases. Lowercase letters show differences across interacting pairs within the same group size.*

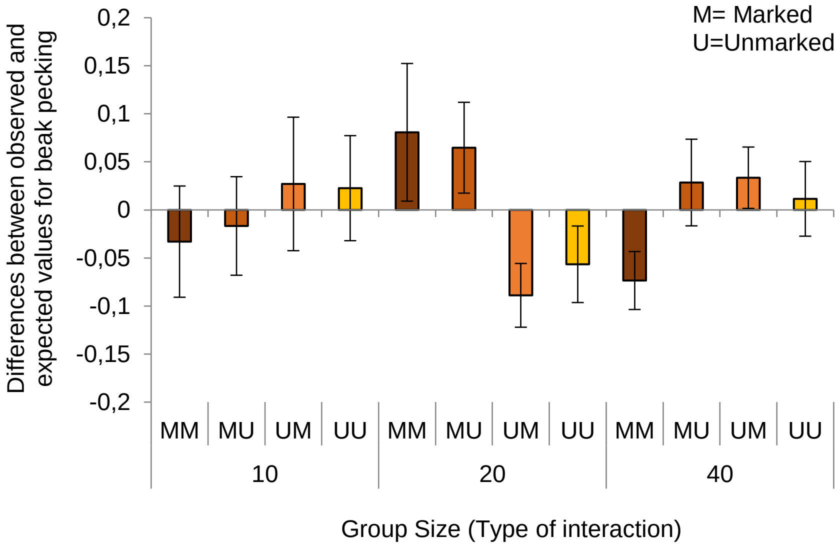


Figure 2.4 *Differences between observed and expected values for beak pecking pecks. No significant differences were found across different group sizes or interacting pairs when using Bonferroni post-hoc comparisons.*

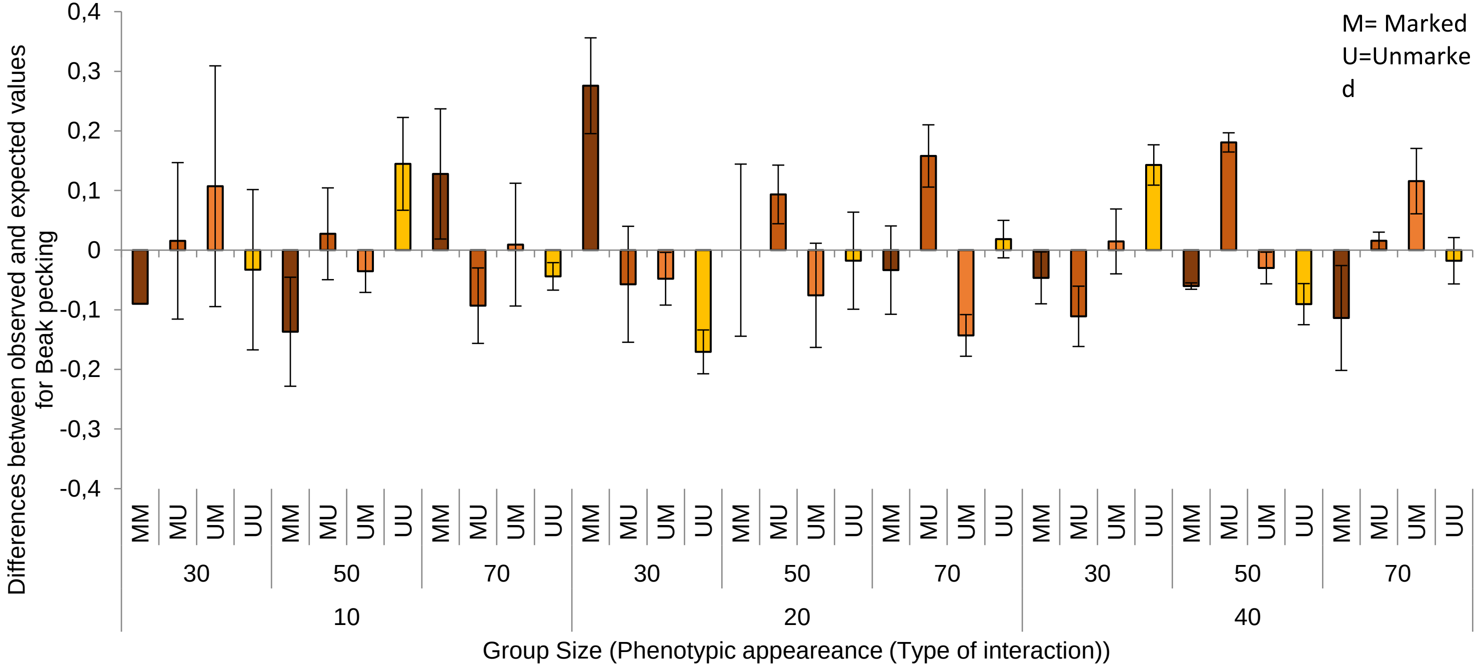


Figure 2.5 *Differences between observed and expected values for beak pecking. Bonferroni post-hoc comparisons showed no significant difference among groups (P > 0.05).*

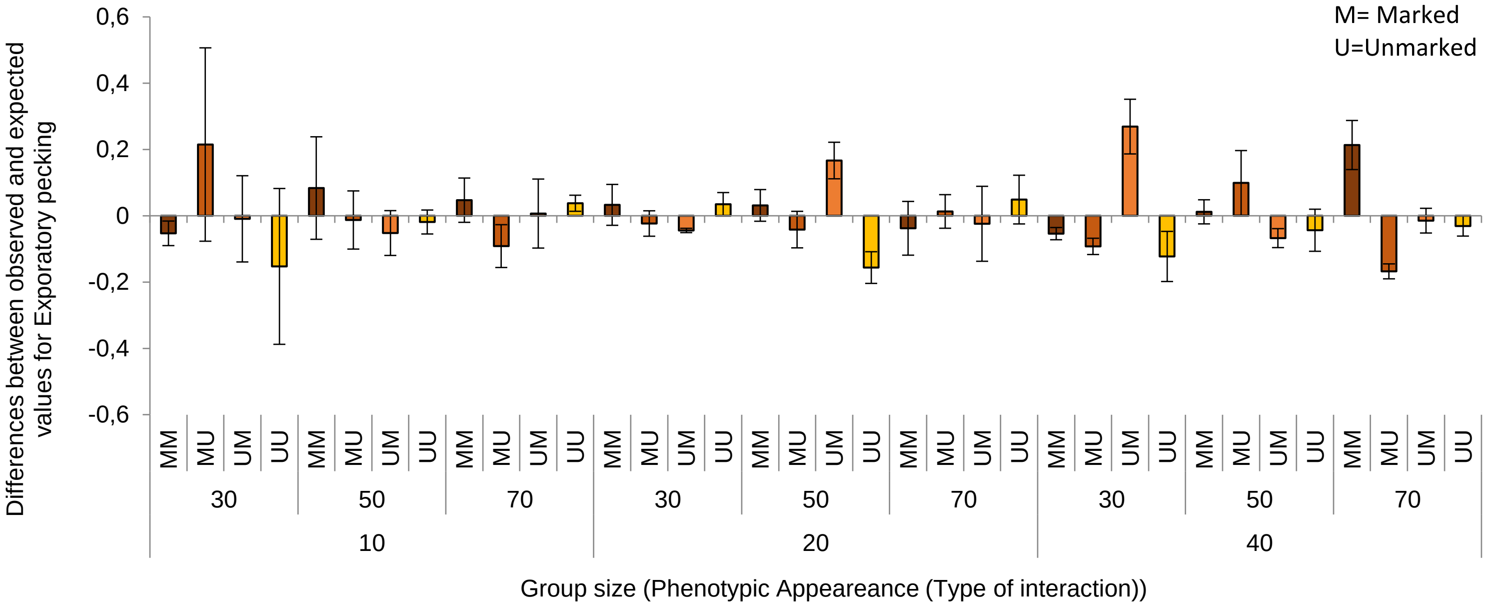


Figure 2.6 *Differences between observed and expected values for exploratory pecking. Bonferroni post-hoc comparisons showed no significant difference among groups (P > 0.05).*

2.5 Discussion

This study investigated whether group size (GS) and alterations of the phenotypic appearance (PA) from day one of different bird proportions in a common layer strain (Hy-Line Brown) affected their social dynamics. Aggressive events were analysed in two categories; threats and the sum of aggressive pecks, leaps and chases. The results of the study showed that most of the aggressive interactions performed by the pullets were in the form of threats, while other types of interactions such as aggressive pecks, leaps and chases appeared at much lower frequencies.

Threats are the cheapest option in terms of energetic demand to assess the fighting ability between conspecifics (Számadó, 2008) and are considered to be a warning signal to other individuals that do not require physical contact (Estevez et al., 2002). Aggressive pecks, leaps and chases, on the other hand, are considered active aggressive interactions as they may involve physical contact, imply a risk of suffering injuries or require high activity levels. Thus, it is not surprising that this form of less costly aggressive interaction was the most frequently observed during the study period in which a higher number of interactions are expected (Rushen, 1982).

As hypothesised, threats were performed at higher frequencies in small (10) than in large GS (20–40). However, no differences were detected for the sum of aggressive pecks, leaps and chases. These results are similar to those found by Estevez et al. (1997) who also reported a decrease in the frequency of threats as GS increased in broiler chickens, but no effect on costly fights. Therefore, our findings provide additional support to the hypothesis that in large groups social dynamics are based in a tolerant system (Estevez et al., 1997), probably because the cost of establishing a hierarchical system outweighs the benefits (Pagel and Dawkins, 1997). Other studies in laying hens also found evidence of low frequency of aggressive interaction in large groups of laying hens (Hughes et al., 1997 (300–700 birds); Nicol et al., 1999 (72–168, 264); Estevez et al., 2002, 2003 (15–120); D'Eath and Keeling, 2003 (10–120)).

Interestingly, the analysis of affiliative behaviours such as exploratory pecking and beak pecking yielded a similar response to variations in GS, which in both cases decreased as GS increased. It has been proposed that allopreening, or in our case, exploratory pecking, might replace aggressive behaviour in individuals that are forced to live in close proximity (Harrison, 1965), or play an important role in reducing aggression levels when social bonds are being established (Sparks, 1964). Gentle feather pecking has also been described to have an important social exploration function that might be of particular relevance during early rearing (Zajonc et al., 1975; Riedstra and Groothuis, 2002). Therefore, even though the period of study is larger in our experiment (3–13 weeks of age), according to the hypotheses of Riedstra and Groothuis (2002) affiliative behaviours such as exploratory pecking, and perhaps beak pecking, should be expected to increase in larger GS. On the contrary, our results show a linear reduction of both types of affiliative interactions as GS increase.

Zimmerman et al. (2006), argued that laying hens in small commercial flocks (2450 birds) showed higher levels of aggressive interactions, as well as preening and allopreening, which was explained as an effect of the lower availability of space as compared to larger flocks (4200 birds), although no spatial data were collected in the study to corroborate this relationship. In this respect, Liste et al. (2015), using data from focal observations on a sample of the same pullets used in the current study, showed that despite controlling for the effects of density (8 birds/m^2), birds in the largest groups of 40 had higher levels of locomotion and travelled longer total, net and maximum distances as compared to pullets in groups of 10 and 20. Such results were explained as an effect of the larger resulting pen size in larger groups as relative space available increased proportionally with GS as space allotted per hen can be shared by group members allowing a greater freedom of movement. In a study specifically designed to determine the differential effects of GS, density and pen size on use of space Leone and Estevez (2008) concluded that pen size was the factor with the most impact on parameters characterizing movement and use of space in broiler chickens. A study in sheep (Averós et al., 2014) also showed that ewes maintained at constant GS, while varying density from 1 to 3 m^2/ewe, had higher frequencies of both, positive and negative social interactions at high density of 1 m^2/ewe in comparison to individuals maintained at 2 or 3m^2/ewe. They attributed these results to the fact that a lower space availability would increase the chances of finding other individuals in their path of movement, thus promoting higher level of interactions both negative in the form of increased aggressive interactions, as well as positive affiliative within the group, as found in our current study in the smallest pens. Taking all this into consideration, it is possible to suggest that the frequency of affiliative and aggressive social interactions would be the result of the probability of encountering another individual that would depend on density and pen dimensions.

Other possible explanations for the results obtained are that group cohesion may decrease as groups become larger as found in primates (Henzi et al., 1997a, b), possible due to a limitation in cognitive capabilities (Dunbar, 1992), or to time constrains needed for developing social bonds (Lehmann et al., 2007). Time spent grooming plays an important role in maintaining social bonds in primates (Lehmann et al., 2007). Thus, if group cohesion in pullets is reduced with increasing group size because they function under a tolerant system, a similar reduction in affiliative interactions might also be expected. Classical studies suggested that the domestic fowl is able to individually recognize up to 96 birds (Guhl, 1968), therefore our experimental groups would not be considered too large to be able to establish some kind of social structure. However, no evidence of the latter was found as neither aggressive nor affiliative behaviours were found to increase with GS as would be expected. Other factors no tested here such as age or total space availability may have affected to the results obtained (Liste et al., 2015).

In this study it was predicted that social dynamics would not only depend on the size of the group in which pullets were reared, but also on the phenotypic diversity of their social environment (0, 30, 50, 70 and 100% of PA alteration). This hypothesis was based on the results obtained by Estevez et al. (2003) and Dennis et al. (2008) which suggested that phenotypically altered birds would be at higher risk of being targeted in aggressive encounters. In addition, Dennis et al. (2008) found that such risk was frequency dependant: birds presenting an altered phenotype were at a higher risk to become targets of aggression when they represented a low proportion within the population. Contrary to our expectations, we did not detect any effect of PA treatment or PA × GS interaction for any type of aggressive or affiliative behaviours considered in this study. The birds were one-day old when they were phenotypically altered and exposed to their group mates. During the first hours after hatching chicks imprint with their mother, but also with their siblings (Queiroz and Cromberg, 2006) and are able to recognize them even though feathers and other physical characteristics change with age (Bateson, 1979, 1990). Thus, it is possible that the birds in our study would have assumed the diversity of the phenotypes (or lack of it) by imprinting or by early filial learning processes (Bolhuis and Bateson, 1990; Bolhuis and Honey, 1998) that could facilitate group mate recognition. However, Dennis et al. (2008) also manipulated the birds' phenotype on day one, but they found clear effects in the frequency of aggressive interactions received that also had a strong repercussion on the body weight of altered broiler chickens.

A second possible explanation for the lack of effects of the PA treatment may have been the relatively low conspicuity of a black mark over brown plumage coloration, while in both, Estevez et al. (2003) and Dennis et al. (2008), the marks were applied over white birds. McAdie and Keeling (2000) removed and manipulated feathers in Lohmann Brown laying hens. The manipulated areas became more visible as they exposed the white inner feathers or their skin triggering a feather pecking response. Therefore, it is possible that if the mark used in our study was not sufficiently conspicuous, or if it did not created sufficient contrast against the background coloration to be perceived as something different it may not have had a strong enough effect to be socially explored or pecked, or at least not with the sufficient intensity to be able to detect an impact of such alteration. It may be questioned if that perhaps the tags were distracting from the effects of the marks. However, all birds irrespectively of their phenotype carried individual tags of identical dimensions and colours. In this study as well as in Dennis et al. (2008) minimal tag pecking was observed.

But despite the lack of effects of the PA treatments, some interesting results were found when analysing the directionality of the interactions when considering all possible alternatives (MM, MU, UM, UU) in heterogeneous populations (30, 50, 70%). We did detect a trend of type of interaction for the sum of aggressive pecks, leaps and chases (P = 0.07), showing a tendency to be higher than expected from unaltered (U) directed towards altered (M) pullets. For all other types of interaction (MM, MU, UU), their frequency was negative, indicating a lower than expected rate of interactions. In addition, the significant effect of GS by type of interaction for the sum of pecks leaps and chases showed that in GS 20 there was a significantly higher than expected frequency of interactions from U towards M birds (Fig. 2.3). These results suggest that active aggressive events, although being less frequent than threats at early ages, seem to be more directed towards individuals with altered phenotypes, and that phenotypic differences appeared to be more important on determining the type of social interactions in intermediate GS (20 individuals). Thus, these results suggest a non-random directionality of active aggressive interactions from U towards M birds that was clear in

GS20, similar to the finding by Estevez et al. (2003) and Dennis et al. (2008). Interestingly, when analysing the impact of the PA treatment on the same group of birds, Marin et al. (2014) reported significant lower body weights at 24 weeks of age in the 30% treatment, the group in which the altered phenotype should have had the highest impact. Altered birds in these groups were around 150 g lighter as compared to the body weight of their 70% unaltered counterpart within the same pen. However, this effect was only observed in the smallest experimental GS10.

According to Dennis et al. (2008) aggression is expected to be directed towards individuals with the less common phenotype. From our results, it appeared that in addition to the proportion of individuals showing a determined phenotypic appearance (in intermediate populations), the number of individuals in a group carrying the alteration are also important in modulating its effect. The larger number of altered birds in GS 40, even in the 30% treatment, may have diluted its impact, explaining why no effects were found in the large groups in this study or in Marin et al. (2014) prior to later modifications of the phenotype in adulthood. In addition, it is essential to indicate that despite the fact that the observation methodology was designed to collect as many as possible aggressive interactions occurring in a pen and the phenotypes of the birds giving and receiving the interaction, the low frequency of aggressive interactions and the large number of pens to be observed (a total of 45 pens) may have prevented us from detecting clearer evidence for the effects of PA as well as in their interactions with GS.

An interaction between the effects of GS by the type of interaction was detected for beak pecking. Although, the Bonferroni post-hoc comparisons were not significant we could observe a higher level of beak pecking from M birds toward M and U birds in groups of 20 as compared to all other possible types of interaction. Interestingly M birds were clearly targeted in GS 20 as indicated earlier. Thus, it is possible to speculate based on this tendency that perhaps M individuals in the more socially unstable groups of 20 birds would tend to perform more beak pecking as a form of submissive behaviour in order to reduce the negative effects of being target of active aggression.

Also, an effect of the triple interaction between GS, the phenotypic treatment and the type of interaction were detected for beak pecking and exploratory pecking. However, the Bonferroni posthoc comparisons were not significant and not clear trends appear to be evident. It has been described that individuals that are phenotypically similar tend to cooperate more (Antal et al., 2009), however the results of this study do not appear to support this hypothesis for the moment. It is possible that the combined effects of GS, PA treatment and type of interaction may have some influence on the social interactions and social bonding but perhaps a more exhaustive data collection it is necessary to be able to detect these subtle effects.

Social dynamics in groups of domestic fowl are based on the direct assessment of individual's status (Wood-Gush, 1971) and would require to individually recognize their pen mates (Bradshaw, 1992) and remember their social status (Wood-Gush, 1971; Rushen, 1982). However, individual recognition might not be possible in large groups (McBride and Foenander, 1962; D'Eath and Keeling, 2003). In fact, birds at intermediate group sizes of 30 individuals had reduced body and egg weight probably as result of social disruption because groups were too large to form a stable hierarchy but not sufficiently large for a tolerance system (Keeling et al., 2003). When the formation of a hierarchy is not possible, badges of status, such as coloured feather patches may determine the type of interaction that would be established between interacting pairs. Birds in this intermediate GS may decide if they should fight or avoid a conflict depending on the direct assessment of the physical characteristics of the opponent (Estévez et al., 1997; Pagel and Dawkins, 1997; D'Eath and Keeling, 2003). Thus, we argue that at intermediate groups in which individual identification becomes troublesome birds would pay special attention to badges of status and thus phenotypic traits may become more relevant in such conditions to identify the potential fighting ability of the opponent.

From the applied stand point these results can help on understanding how social dynamics of pullets depended on the size of the group, but also, how relevant is the effect of the phenotypic appearance, effects that were not investigated before. These results may help, for example in understanding the reasons why birds showing an altered phenotype, due to disease, injury or other factors may result in targeted aggressive interactions towards such individuals. This knowledge might help farmers to detect individuals that could easily become victims of aggression and prevent them from major welfare problems.

2.6 Conclusions

This study shows that birds from small groups showed higher levels of aggressive and affiliative social interactions in comparison to larger groups. This could be the result of the lower total space availability when birds are housed in small groups (even when density remains constant), or due to the establishment of a hierarchical social system that is determined during this rearing period (implying a higher rate of interactions) in small groups but not in larger groups as they will tend to build a more tolerant system. Nevertheless, as both affiliative and aggressive interactions seem to follow similar patterns with increased GS, it is possible that the frequency of interactions is strongly modulated by the probability of running into another individual. Our results also revealed that in heterogeneous groups active aggression was directed from U to M birds at intermediate GS 20. This could be explained as a consequence of the breakdown of the hierarchical system at intermediate GS which would imply an increased impact of secondary characteristics to assess bird's social status. These results can help on understanding the complex effects of group size and space efficiency on the social dynamics of laying hens.

Acknowledgements

This project was funded by the Ministry of Economy and Competitiveness of Spain (AGL2010-18276, MODELAY) which covered G.L. salary and a postgraduate scholarship for I.C. The authors wish to thank Iurancha González, for her help with the pen construction and animal care throughout the study. Special thanks to I. Beltrán de Heredia and D. Lizaso for their veterinary assistance.

CHAPTER 3:

The looks matter; aggression escalation from changes on phenotypic appearance in the domestic fowl.

3.1 Abstract

Domestic fowl in small groups are assumed to establish hierarchical systems based on individual recognition. Conversely, interactions in large groups are modulated by badges of status. Previous studies suggested that birds differing in phenotypic appearance (PA) became targets for aggression, possibly mistaking altered PA for badges of status. We evaluated the impact of altering PA on 0, 30, 50, 70 or 100% of the birds' house at three experimental group sizes (GS). Tested GS were 10, 20 or 40 (8 birds/m^2*, 3 pens/GS x PA, 45 total). Thus, for each GS we had groups initially homogenous (100U, U = Unmarked; 100M, M = Marked), or heterogeneous M and U phenotypes coexisting in different proportions: 30M/70U, 50M/50U, and 70M/30U, remaining unchanged until 33 weeks of age. Then, homogeneous groups (100U and 100M) were sequentially altered by marking or unmarking 30, 50 and 70% of birds at 34, 38 and 44 weeks, respectively. Aggressive interactions were observed before applying changes at 27–28 weeks (T0), and after each sequential PA change on week 35–36 (T1), 39–40 (T2) and 45–46 (T3). Frequency of aggressive interactions in altered groups at T1, T2, and T3 were compared with non-altered heterogeneous controls. Results indicate no differences across initial PA and GS treatments (T0; P>0.05). However, aggression escalation was observed at T1 immediately after the first PA manipulation (Tukey P<0.05 altered vs controls). Aggression in altered groups remained high at T2 when compared to controls (Tukey, P<0.05), although by T3 interactions declined to almost initial levels (Tukey, P>0.05 altered vs controls). Aggressive interactions at T1 and T2 were predominantly directed from un-altered towards*

recently altered birds, irrespectively of their initial phenotype and of the GS. These results demonstrate that a sudden change in PA affects group dynamics. Altered birds were exposed to escalated aggression even in small groups, where individual recognition was presumed.

3.2 Introduction

The domestic fowl is a social species that when in small groups forms a stable dominance hierarchy or ´pecking order´ that is established through aggressive interactions (Schjelderup-Ebbe, 1922; Wood-Gush, 1971; Rushen, 1982). Once a stable hierarchy is formed, aggressive interactions are replaced by dominance-subordinance interactions (Queiroz and Cromberg, 2006). It is assumed that under this type of hierarchy birds recognize group mates individually (McBride and Foenander, 1962) and remember the outcome of aggressive encounters (Cloutier et al., 1995).

The frequency and intensity of aggressive interactions to form a stable hierarchy are group size dependent (McBride and Foenander, 1962; Al-Rawi and Craig, 1975; Al-Rawi et al., 1976; Hughes and Wood-Gush 1977), as higher number of interactions among group members would be required to establish dominance relationships in larger groups. In addition, remembering the outcomes of all occurring interactions within a large group can be challenging, leading to less stable social structures. Similarly, increased aggression during hierarchy formation seems to occur only when group sizes remain relatively small, while in larger groups aggressive interactions are lower than expected (Estevez et al., 1997; Hughes et al., 1997; Nicol et al, 1999). These results led to consider that domestic fowl living in large groups were likely to base their social relationships in a more flexible, tolerant system (Estevez et al., 1997; Estevez et al., 2002; Estevez et al., 2003).

With regard to negative impact on poultry production, the most conflicting group size appears to be intermediate sized groups, as opposed to larger groups (60 or 120) as described by Keeling et al., 2003. These authors proposed that group sizes around 30 birds could represent the turning point between establishing a hierarchical system typical of small group sizes (Collias and Collias, 1996; Mench and Keeling, 2001), to a tolerant social system better suited for large group sizes. Pagel and Dawkins (1997), provided the mathematical frame to explain this social plasticity by showing that trying to form a hierarchy would only be cost effective in a narrow range of (low) group sizes. In large groups, where individual recognition is not feasible, social interactions would be modulated through badges of status (Pagel and Dawkins, 1997).

Although, olfactory and auditory cues may help individuals in social contexts (Jones and Faure, 1982; Temple et al., 1984, Jones and Gentle, 1985) social discrimination in the domestic fowl seem to be based on visual cues (D'Eath and Stone, 1999; Hauser and Huber-Eicher, 2004). In fact, visual cues such as comb and wattle size and colour, body size or plumage colour, all are known to provide important information regarding the health status (Zuk et al., 1990), fighting abilities and competitive potential of the domestic fowl (Marks et al., 1960; Siegel and Hurst, 1962; Cloutier et al., 1996; Martin et al., 1997; Cloutier and Newberry, 2000). Among chickens, and numerous wild bird species such as house finches (*Carpodacus mexicanus*) or Eurasian siskins (*Carduelis spinus*), status signals are often presented as ornamental traits located around the head and neck area (Zuk et al., 1990; Belthoff and Gautheraux, 1991; Senar et al

1993) which highlights the relevance of this body area in the assessment of social contests. Considering that domestic fowl use visual cues to assess individuals’ competitive ability, it is not surprising that aggression is generally directed towards individuals presenting a discrepancy from the flock ´normal´ phenotypic appearance, including changes in plumage coloration (Estevez et al., 2003; Dennis et al., 2008; O’Connor et al., 2011).

Other possible explanation may involve more complex evolutionary processes like kin selection (Hamilton 1964 a, b) or, alternatively, phenotype matching (Grafen, 1990). It is speculated that phenotypic appearance may serve to identify the degree of kinship and would explain why individuals with similar appearance would tend to cooperate and interact less aggressively (Hamilton 1964 a, b; Lizé et al., 2006). However, discrimination may also arise through a more parsimonious mechanism, phenotype matching. Phenotype matching would permit group-member and species recognition (Grafen, 1990; Barnard, 1991) by learning the phenotype of familiar relatives, or of oneself (self-referent phenotype matching). Through this mechanism, animals would form a phenotypic template to compare against the phenotypes of familiar and unfamiliar individuals (Mateo and Johnston, 2000; Gerlach and Lysiak, 2006). Phenotype matching may also help recognizing unhealthy individuals that can bring a significant risk to the population (Zuk et al., 1990; Hughes and Cremer, 2007). Thus, phenotype templates would facilitate the identification of unrelated individuals that could out-compete locals for valuable resources, or of sick ones that could be vectors of diseases to the local population.

Phenotypic templates are formed early during post-natal development in precocial birds, based on the existing phenotypes in their social environment (Gerlach and Lysiak, 2006). When more than one phenotype co-exists, it is logical to expect that birds would tend to identify themselves with the most common phenotype in the group. It could be speculated that diversity in phenotypic appearance occurring early in life would be easy to incorporate into the group social dynamics. Contrarily, the emergence of new phenotypes in adulthood, once the template is well-established, would be expected to be much more disruptive.

Commercial flocks of domestic fowl such as laying hens have been genetically selected for performance and homogeneity in a wide range of parameters (body weight, feather colour, sexual maturity, eggshell colour, egg weight (Hocking et al., 2003)) and management practices are design to maintain bird homogeneity. However, phenotypic variability may emerge during the production cycle as a result of individual differences on development, feed intake, health status or injuries, among other factors. In alternative production systems phenotypic variability may be due to the use of mixed lines to maintain local breeds, or to offer a wider variety of products (e. g. white and brown eggs Leenstra et al., 2012). Thus, phenotypic variability may be relevant for the welfare, health and performance of these flocks.

In a previous study we investigated the effects of altering the phenotypic appearance (PA) of different proportions of birds (0, 30, 50, 70, 100% of birds altered) upon arrival to the experimental facility at one day of age (Liste et al., 2015; Campderrich et al., 2017). The birds were maintained at three experimental group sizes (GS; 10, 20 and 40 individuals) during the rearing period. In these studies, a larger number of social interactions, aggressive and affiliative, were found in small groups of 10 compared to groups of 40 (Campderrich et al., 2017), while locomotion was higher in larger group (Liste et al., 2015). By contrast, the effects of PA were unclear, although there was some indication that aggression was mainly directed from unmarked (U) towards marked (M) birds irrespective of the proportion of U or M individuals in each group (Campderrich et al., 2017).

In this follow up study we investigated the impact of manipulating the phenotypic appearance in adult domestic fowl (Hy-Line Brown). We hypothesized that manipulation of the phenotypic appearance in socially stable groups of adult birds will produce a sudden increment (escalation) on aggressive interactions. We predicted that the impact of such manipulation will be smaller in small groups where individual recognition is assumed (e.g. 10 birds) as compared to larger groups where individual recognition is less likely. It was also predicted that the response to the alteration of the phenotype will be stronger the lower the proportion of altered birds and that aggression will be specifically directed towards recently altered birds.

3.3 Material and methods

This project was approved by the Ethical Committee at Neiker-Tecnalia and the Livestock Services of the Regional Government (Diputación Foral de Alava, permit number CEE_2010_002), complying with the Spanish legislation regarding the use of animals for experimental and other scientific purposes (Real Decreto 1201/2005). This study was part of a larger project that evaluated different aspects of phenotypic appearance and group size on the welfare, health and performance of laying hens.

3.3.1 Animals and housing conditions

1050 one day old laser beak-trimmed female chicks of a laying strain (Hy-Line Brown) were obtained from a commercial hatchery (Avigan-Terralta, Tarragona, Spain). They were transported to the experimental poultry facility in Neiker-Tecnalia (Vitoria-Gasteiz, Spain). The facility contained 45 experimental pens that were constructed with PVC piping and plastic netting. Pen walls were shielded with a dark plastic to prevent visual contact across pens. Pen floors were covered with 1.5 Kg/m^2 of wood shavings. Drinking (1 nipple drinker/5 birds) and feeding space (4cm/bird) in each pen was proportional to the number of birds housed. Birds were fed ad libitum with a commercial diet according to their rearing stage. Lighting, temperature and ventilation were controlled with a computerized system and followed standard commercial practices. Ambient temperature at arrival of the chicks was 36ºC and was progressively decreased according to standard management practices until reaching 18-20ºC at six weeks of age. After that, temperature was maintained through the study. The lighting programme was also standard; 24 h of light provided the day of arrival which was progressively reduce to reach 9h at 14 weeks of age. Photo-stimulation started at 15th weeks to reach 16h light/8 h dark at the onset of lay (first egg laid 16th week of age). This photoperiod was maintained during the experiment (27 to 46 weeks). At 14 weeks of age, before the onset of lay, pens were furnished with nests and perches according to national legislation (Directive 1999/74/CE, Real Decreto 3/2002).

3.3.2 Experimental design

Preliminary studies were conducted with these birds during their rearing phase (Liste et al., 2015; Campderrich et al., 2017). During these initial studies, one day old chicks were randomly allocated to one of the 45 experimental pens housing 10, 20 or 40 birds (N = 15 pens per GS). In order to maintain a constant density (8 birds/m^2), pen sizes were adjusted to GS: 0.75 x1.78 m (1.25 m^2), 1.00 x 2.50 m (2.5 m^2) and 2.00 x 2.50 m (5 m2), for GS 10, 20 and 40 birds, respectively. GS treatments were combined in a full factorial set up with 5 different initial phenotypic appearance (PA) treatments. The PA treatments consisted on the manipulation of the appearance of different proportions of birds within each group (0, 30, 50, 70 or 100%, N = 9 for each PA treatment). Consequently, two types of groups were formed: homogeneous populations were all group members were either unmarked (100U) or marked (100M) and heterogeneous populations were M and U birds coexisted in the same pen but at different proportions (30M/70U, 50M/50U, 70M/30U). Each GS by PA combination treatments were replicated in 3 pens.

The PA alteration consisted of a black mark made with a non-toxic dye that covered the feathers on the back of the birds´ head (Estevez et al., 2003; Dennis et al., 2008); see Fig. 3.1. To maintain PA treatments during growth, marks were reapplied as needed (every 3 to 6 weeks, up to 20 weeks of age). Marks were made as similar as possible and were always performed by the same team that agreed in the location and area covered that was proportionate to the growth of the birds. In addition, each bird was individually identified by two laminated paper tags attached to the sides of the neck (Fig. 3.1) following procedures as described in

Cornetto and Estevez (2001a). The tags included the pen number and the bird ID (for further details see Campderrich et al., 2017). These tags were displayed by all birds (both M and U) so their effects on PA were standardized. Additionally, previous research found that pecking at the tags decreased to negligible levels after the first week due to habituation (Dennis et al., 2008) suggesting that the tagging did not interfere with the effects of the PA treatments applied.

Figure 3.1 *Photo description of the two PA treatments used in this experiment. M (presenting Marked individuals), U (presenting Unmarked individuals).*

The birds remained under the above experimental conditions until the onset of the current phase of the study, when manipulations over homogeneous groups (100M and 100U) took place on the adult birds. The first PA alteration took place at 34 weeks of age by randomly marking (100U) or unmarking (100M) 30% of the birds per pen. The marking was performed as explained above. Unmarking was achieved by applying an H_2O_2 solution to the originally black coloured feathers (Marin et al., 2014), returning them to their natural brown coloration. After this procedure, 100U groups changed to 30M/70U (30M being the recently altered subgroup), while 100M groups were converted into 70M/30U (30U being the recently altered subgroup). The second PA change was applied at 38 weeks, with an additional alteration to 20% of the birds per pen. This resulted in pens with a 50M/50U composition: half of them where 50M were originated by marking from 100U, and the other half where 50U resulted by unmarking from 100M. Finally, the 3rd PA change was applied at 44 weeks where an extra 30% of birds per pen were altered. This resulted in the final groups of 30U/70M (originally 100U) and 30M/70U (originally 100M). See Table 3.1 for a detailed description of the experimental design**.**

3.3.3 Data collection

Direct behavioural observations of each pen were carried out by the same observer between 8:30h and 14:00h. The observations were conducted during two consecutive weeks for each time period, starting prior to the birds´ manipulation (T0, weeks 27–28). Then, observations took place after each PA change at T1 (weeks 35–36), T2 (weeks 39–40) and T3 (weeks 45–46). Once the changes were introduced in homogeneous populations, we waited for three days before starting observing the birds.

Table 3.1 *Experimental design. Three different group sizes (GS) were tested (10, 20 and 40) for each original phenotypic appearance (PA) treatment: 100% U (100U), 30% (30M/70U), 50% (50M/50U), 70% (70M/30U), 100% M (100M)). U: Unmarked, M: Marked. Originally heterogeneous groups: 30, 50 and 70% altered from day one were used as controls. Adapted from Marin et al., 2014.*

		Weeks of age (observation period)				
		27-28 (T0)	**35-36 (T1)**	**39-40 (T2)**	**45-46 (T3)**	
	Group Size	***Original groups***	***1st PA change (30% altered)***	***2nd PA change (50% altered)***	***3rd PA change (70% altered)***	**# Pens**
Homogeneous Groups (Sequentially altered)	10	0% Marked	30% Marked 70% Unmarked	50% Marked 50% Unmarked	70% Marked 30% Unmarked	3
	20					3
	40					3
	10	100% Marked	70% Marked 30% Unmarked	50% Marked 50% Unmarked	30% Marked 70% Unmarked	3
	20					3
	40					3
Heterogeneous Groups (Controls)	10	30% Marked 70% Unmarked	30% Marked 70% Unmarked		30% Marked 70% Unmarked	3
	20					3
	40					3
	10	50% Marked 50% Unmarked		50% Marked 50% Unmarked		3
	20					3
	40					3
	10	70% Marked 30% Unmarked	70% Marked 30% Unmarked		70% Marked 30% Unmarked	3
	20					3
	40					3
		45 pens observed	36 pens observed	27 pens observed	36 pens observed	45

During the first set of observations (T0) all 45 pens were observed to determine the basal levels of aggressive interactions occurring in original groups. The number of pens observed afterwards varied as we focused on collecting data from the recently altered groups (originally 100M and 100U) and their corresponding controls. Thus, after the first PA alteration (T1) we observed a total of 36 pens, corresponding to 30M/70U and 70M/30U groups originated from initially homogeneous groups and their controls. For T2 a total of 27 50M/50U pens were observed (recently altered and controls). Finally, 36 pens were observed after the third PA alteration (T3) corresponding to 30M/70U and 70U/30M (recently altered and controls). See Table 3.1 for a summary of the treatments and comparisons performed at each age period.

For each time period (T0 to T3) 10 min direct continuous behavioural observations were collected four times for each pen (40 min total observation per pen) in two weeks period. Pen order observation was randomized. During data collection the same observer sat quietly outside the pen and waited until the birds resume normal activity before starting behavioural observations. The birds were habituated to the regular presence of observers from one day old as they had participated in a previous lengthy behavioural study. All aggressive interactions, fights, threats, aggressive pecks, chases and leaps (according to Estevez et al., 2002, see ethogram Table 3.2) were recorded. The Observer software (V 10.0, Noldus) was used to collect data from each interaction, including phenotypes and IDs´ of the specific individuals interacting. However, when recording the birds´ ID was not possible, due to the bird´s position or in the rare event of several interactions occurring simultaneously, at least the phenotypes of the interacting pair were always recorded. Thus, it was possible to calculate the frequency of interactions between the different phenotypes in each pen, with four possible combinations: MM from marked to marked; MU from marked to unmarked; UM form unmarked to marked; UU from unmarked to unmarked).

Table 3.2 *Ethogram defining the aggressive interactions recorded: aggressive pecks, chases, leaps, threats and fights.*

Ethogram for aggressive interactions (Adapted from Estevez et al. 2002)	
Aggressive peck (event)	The bird raises its head and vigorously stabs its beak towards another bird (usually directed to the head and neck region).
Chase (event)	The bird runs after another bird for at least three steps in an aggressive manner.
Leap (event)	The bird jumps and kicks its feet forward towards another bird.
Threat (event)	The bird stands with head clearly raised (sometimes accompanied with raising of the neck feathers) in front of another bird who held its head at a lower level.
Fight (event)	Two birds stand in front of each other threating and delivering pecks to each other in rapid succession, sometimes accompanied by jumps. Peaks, leaps and threats occurring during a fight sequence were not recorded independently.

3.3.4 Statistical analyses

Due to the low incidence of aggression observed at T0, all aggressive interactions (fights, threats, aggressive pecks, chases and leaps) per pen and time period were lumped into one category called total aggression. Total aggression per pen was standardized according to GS, to allow for statistical comparison among groups of different sizes. The resulting data set was analysed using linear mixed models (PROC MIXED) with GS and PA and their interactions as fixed factors and pen as random effect. Sequential phenotype alteration led to different proportions of PA treatments through time, so each time period was analysed separately. Data were log+1 transformed to meet normality and homoscedasticity assumptions. Significant differences across treatments were further analysed using Tukey post-hoc comparisons.

A second set of analyses was performed (always using data standardized according to GS) to determine the changes in aggression levels occurring across time for each specific PA treatment. Linear mixed models were built including GS as fixed effect and time period as repeated measure. In this case a square root transformation was applied to meet the assumptions of normality and homoscedasticity. Tukey post-hoc comparisons were again employed to clarify significant differences across time periods.

A third set of analyses were conducted to evaluate the directionality of the aggressive interactions in each GS and PA treatment. The observed frequency of aggressive interactions per pen and time period was calculated for each possible interacting pair (MM, MU, UM, UU). We then calculated the expected frequency of aggressive interactions per pen and time period, for each possible interacting pair, assuming that aggressive interactions occurred at random. Expected values were calculated considering the frequency of each phenotype in the pen and the interacting probabilities for each possible pair. Lastly, we calculated the difference between observed and expected frequencies. Significantly higher/lower observed than expected values for a particular interacting pair, would demonstrate the directionality of the aggressive interactions. An independent analysis was conducted for each time period (T0 to T3) to compare recently altered groups with their corresponding controls. The linear model used included GS, PA, type of interacting pair and their interactions as fixed effects. However, when non-significant interactions were detected they were removed from the model one by one according to their AICC. Data were log+1 transformed to meet normality assumptions and Tukey post-hoc comparisons were used to detect differences across treatments. All statistical analyses were conducted using SAS 9.3 software package (SAS Institute, Cary, NC, USA).

3.4 Results

The frequency of total aggressive interactions at the onset of the study (T0) was low and similar for all PA ($F_{4,30} = 1.63$, $P = 0.19$) and GS treatments ($F_{2,30} = 0.87$, $P = 0.43$) or their interaction ($F_{8,30} = 0.59$, $P = 0.77$; Fig 3.2A). At T1, after the 1st PA change was applied to originally homogeneous groups, total aggression showed a 3 to 4-fold increment in recently altered as compared to control groups ($F_{3,24} = 44.17$, $P<0.0001$; Fig 3.2B). These differences decreased but were still evident at T2 ($F_{2,18} = 15.74$, $P = 0.0001$; Fig 3.2C). As the proportion of recently altered birds increased by T3, total aggression receded to similar levels as controls. Regarding this last PA change (T3), the main effect of PA still showed statistical significance ($F_{3,24} = 3.95$, $P = 0.02$; Fig 3.2D), but post hoc comparisons revealed no differences among recently altered and control groups.

GS did not affect total aggression per bird until T3 ($F_{3,24} = 3.94$, $P = 0.02$), when birds in GS40 showed higher levels of total aggression than GS10 (Tukey $P<0.05$; 0.69±0.24a, 1.14±0.28ab, 1.58±0.24b; mean ± SE for GS 10, 20 and 40 respectively). The interaction GS by PA did not affect the total aggression per bird ($P>0.05$).

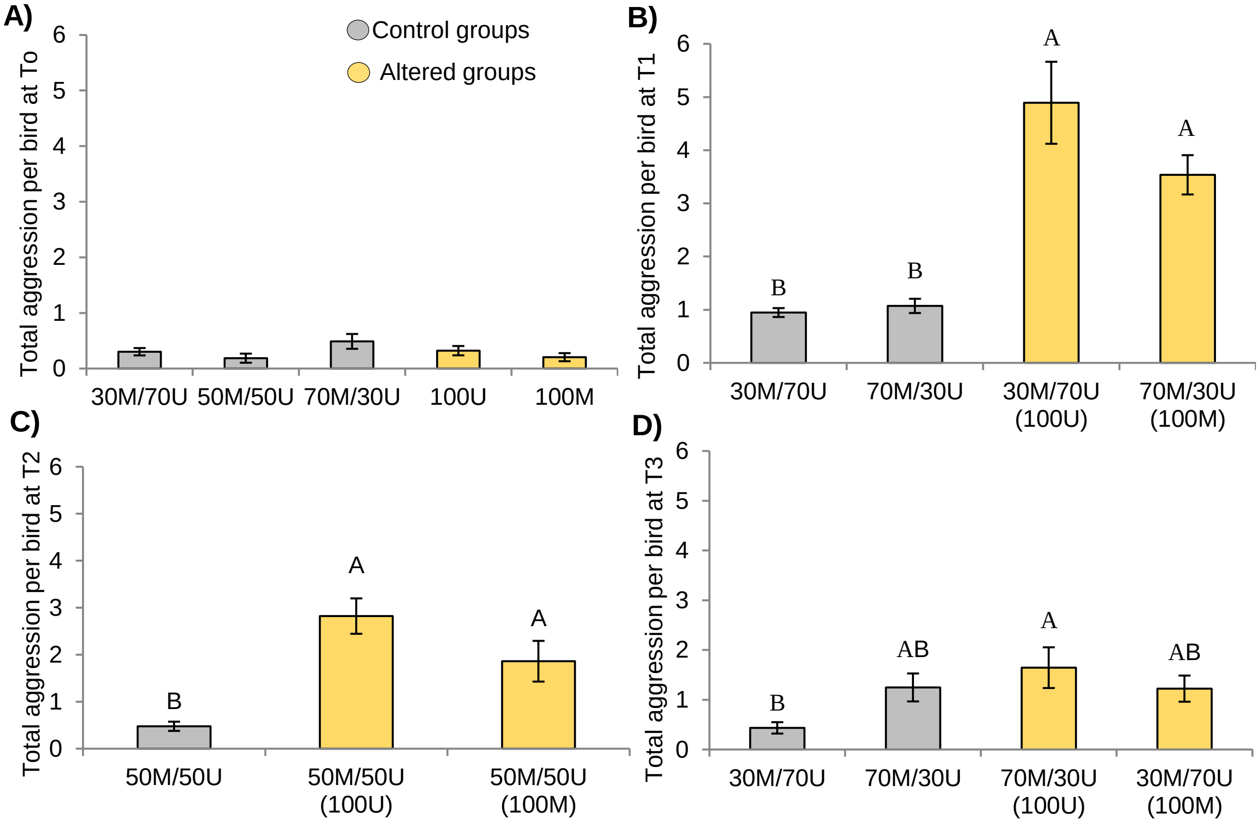


Figure 3.2 *Total aggression (interactions per bird/40 min). Frequency of total aggression per bird at T0 (27–28 weeks; 3.2A), T1 (35–36 weeks; 3.2B), T2 (39–40 weeks; 3.2C) and T3 (45–46 weeks, 3.2D). Bars represent means ± SE. M = marked; U = unmarked. Phenotypic appearance (PA) treatments: originally homogeneous (100U, 100M) and controls (30M/70U, 50M/50U, 70M/30U). Different letters denote significant differences among PA treatments at P<0.05.*

Our second set of analyses showed the impact of sequentially altering PA treatments through T0 to T3 (100M: $F_{3,18}$ = 33.43, P<0.0001; 100U: $F_{3,18}$ = 33.33, P<0.0001; Fig 3.3). Total aggression per bird increased in 30M/70U and 70M/30U control groups at T1, and in 70M/30U control groups at T3, as compared to T0 (30M/70U time effect $F_{2,12}$ = 11.6, P = 0.002; 70M/30U time effect $F_{2,12}$ = 21.5, P = 0.0001, respectively Fig 3.3). This increase occurred even though PA was not altered in these groups. No changes in total aggression were observed for 50M/50U ($F_{1,6}$ = 1.33, P = 0.29; Fig 3.3).

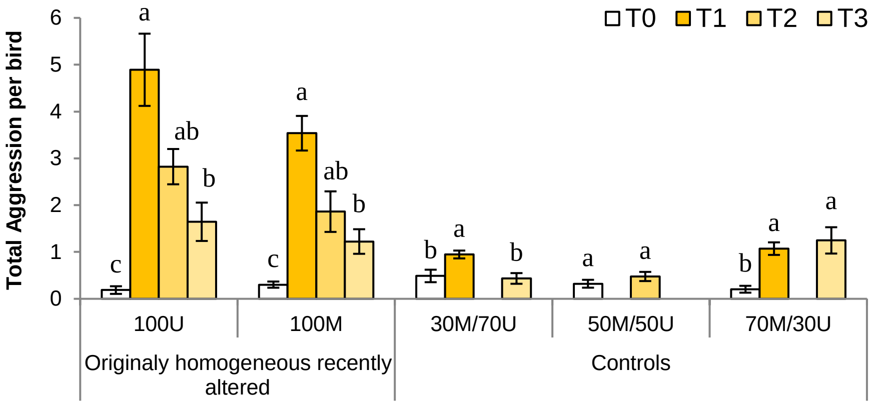
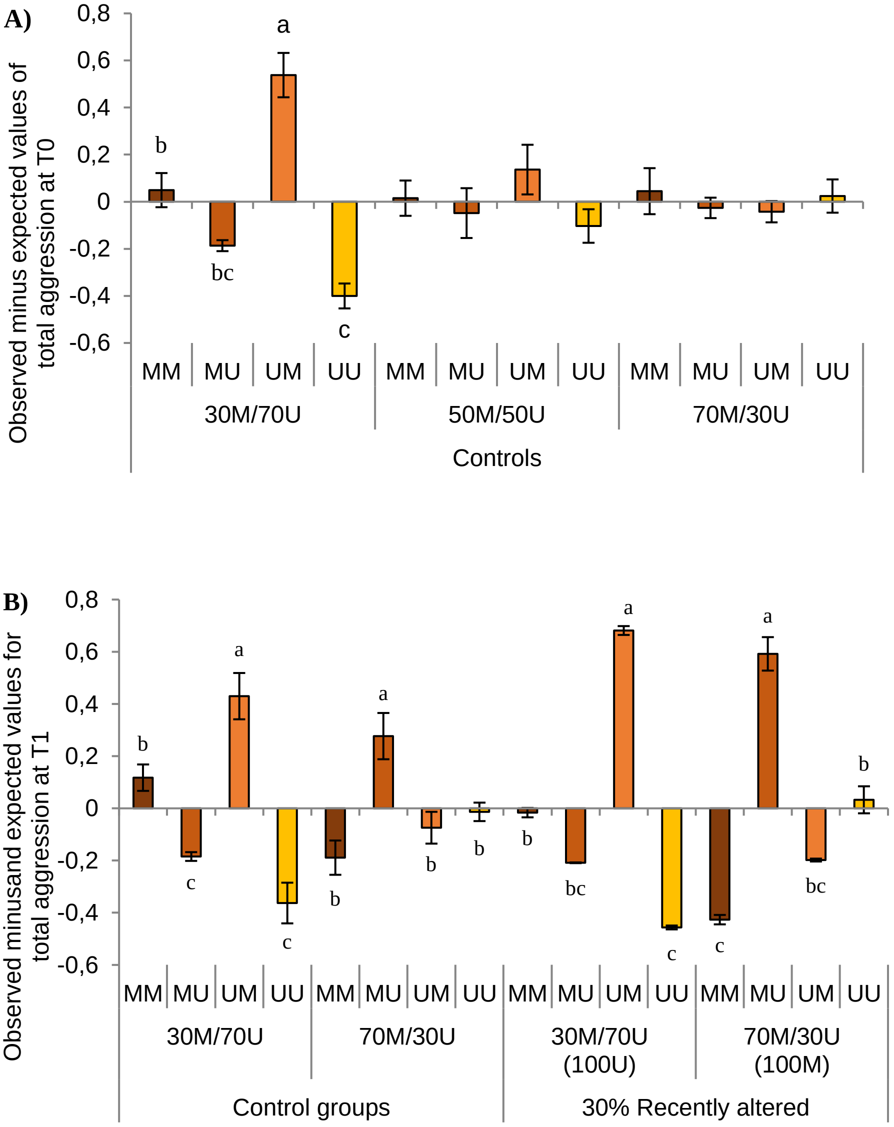


Figure 3.3 *Changes in total aggression (interactions per bird/40 minutes) for each phenotypic appearance (PA) treatment across time. Changes in total aggression per bird for each PA treatment; originally homogeneous groups (100U, 100M) and controls (30M/70U, 50M/50U, 70M/30U), across time periods: T0 (27–28 weeks), T1 (35–36 weeks), T2 (39–40 weeks) and T3 (45–46 weeks). Different letters denote significant differences across time (P<0.05).*

Interestingly, GS did not affect total aggression through the PA changes applied across time ($P>0.05$, all cases). A GS effect was detected only for 30M/70U control groups ($F_{2,6} = 5.43$, $P = 0.045$), where GS20 showed higher total aggression than GS10 (0.83±0.11 and 0.43±0.15, respectively, GS40 showed intermediate values 0.6±0.11). The interaction GS by time period did not affect the total aggression per bird ($P>0.05$).

A clear directionality in the occurrence of aggressive interactions, for each possible interacting pair (MM, MU, UM, UU), was found (PA by interacting pair at T0: $F_{6,64} = 8.95$, $P <0.0001$, Fig 3.4A; T1: $F_{9,88} = 51.43$, $P <0.0001$, Fig 3.4B; T2: $F_{6,64} = 19.69$, $P <0.0001$, Fig 3.4C; and T3: $F_{9,88} = 12.76$, $P <0.0001$, Fig 3.4D). Evidences of directionality were observed at T0 in 30M/70U control groups (Fig 3.4A). Clear directionality of aggression was also found at T1 in 30M/70U recently altered groups and controls (Fig 3.4), with higher than expected interactions from U towards M birds and lower than expected interactions from M towards U birds. Conversely, 70M/30U recently altered groups at T1, presented clear directionality of aggression form M to U birds (Fig 3.4B). Regarding 50M/50U recently altered groups at T2, strong directionality of aggression towards the new emerging phenotypes was also reported: from U towards M birds (initially 100U groups) and from M towards U, (initially 100M; Fig 3.4C). A similar but somehow reduced directionality of aggression was observed at T3 (Fig 3.4D). A significant effect of the interaction between GS and PA was detected at T3 ($F_{18,88} = 2.53$, $P<0.01$; Fig 3.5). The clearest differences on directionality of aggression were observed in 30M/70U control groups at GS 20 and GS 40. No other evidence of directionality was found for either GS 10, 70M/30U control or recently altered groups.



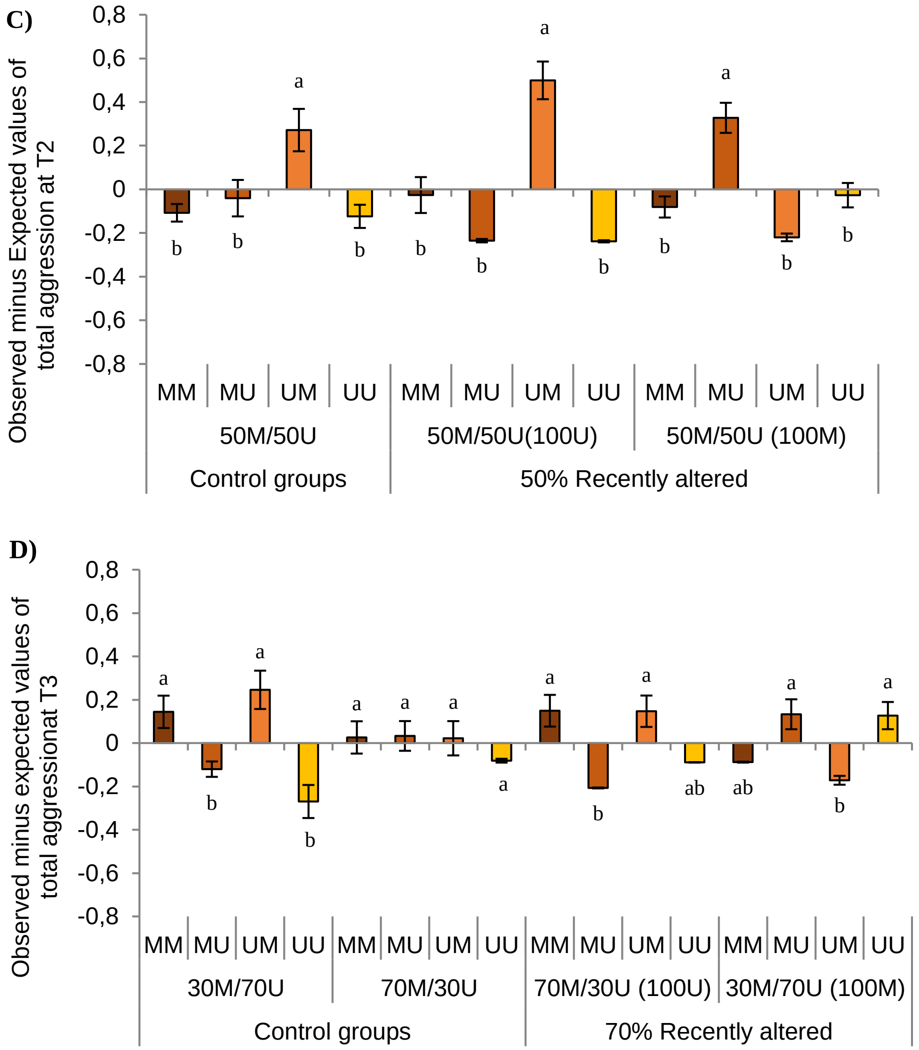


Figure 3.4 *Directionality of aggressive interactions across time. M = marked; U = unmarked. Differences between observed and expected aggressive interactions (means ± SE) for each possible interacting pair (MM, MU, UM and UU) and phenotypic appearance (PA) treatment: originally homogeneous (100U, 100M), and controls (30M/70U, 50M/50U, 70M/30U). 3.4A) T0: 27–28 weeks; 3.4B) T1: 35–36; 3.4C) T2:39–40; 3.4D) T3:45–46. Different letters indicate significant differences among interacting pairs within the same PA treatment.*

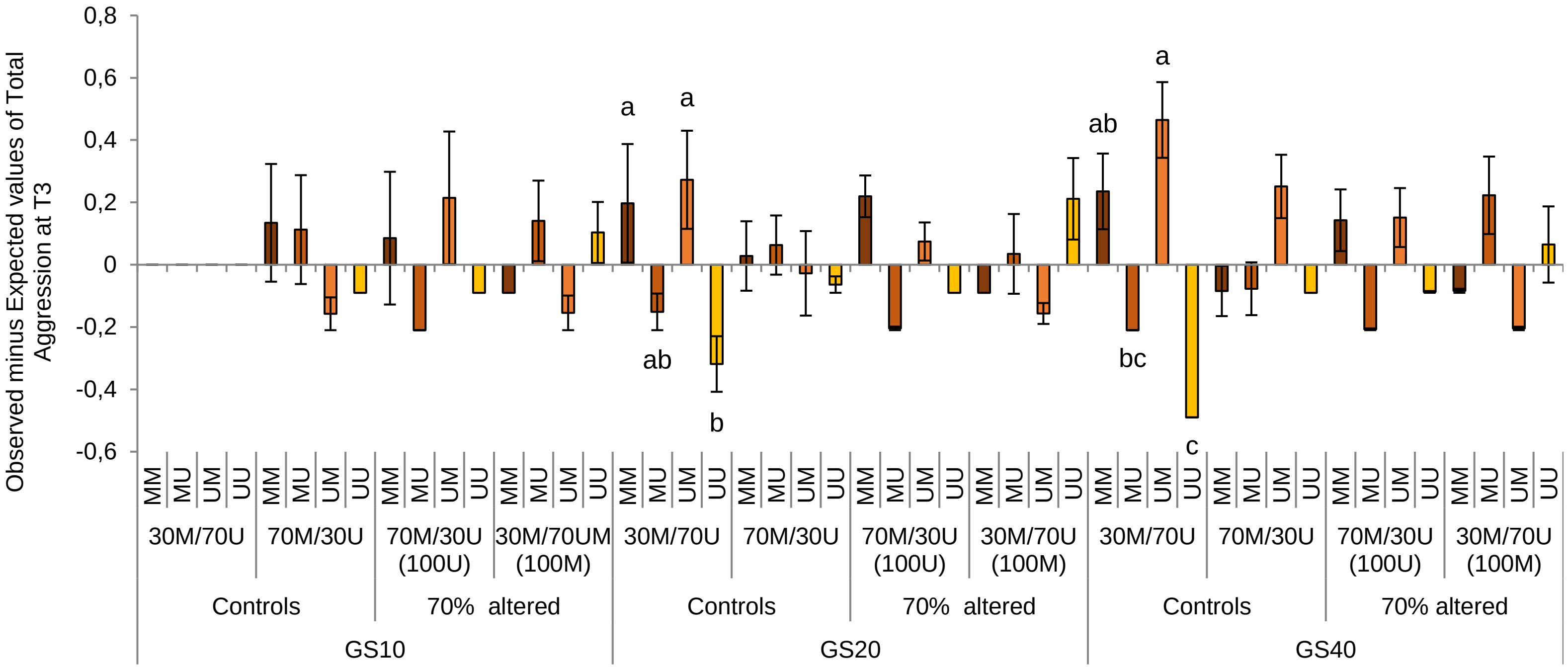


Figure 3.5 *Directionality of aggressive interactions at T3. M = marked; U = unmarked. Differences between observed and expected aggressive interactions (means ± SE) at T3 for each possible interacting pair (MM, MU, UM and UU) and phenotypic appearance (PA) treatment: originally homogeneous (100U, 100M) and controls (30M/70U, 50M/50U, 70M/30U), according to group size (GS 10, 20 or 40 birds). Different letters indicate significant differences, within each GS and PA treatment, for each type of interacting pair (P<0.05).*

3.5 Discussion

The purpose of this study was to determine the effects of sequentially altering the phenotypic appearance (PA) of adult laying hens reared in originally homogeneous groups (100M or 100U) at three different GS (10, 20 and 40). The results of this work showed that the frequency of aggressive interactions was low and similar across all GS and original PA treatments at the onset of the study (T0, Fig 3.2A). Conversely, a substantial increase in aggression was observed at T1 after the 1st sequential PA change was introduced (30% of hens altered in 100U or 100M groups; Fig 3.2B).

Previous studies have shown that domestic fowl can discriminate among group members (Abeyesinghe et al., 2009), have preferences to stay close to familiar individuals (Dawkins, 1982; Bradshaw, 1992) and show aggression to unfamiliar individuals (Guhl and Allee, 1944; Maier, 1964; Craig et al., 1969; Bradshaw, 1992; Dawkins et al., 1995; D'Eath and Stone, 1999). Other studies have also indicated that familiar birds with modified feathers or combs were targeted for aggression when reintroduced in the group (Ghul and Ortman, 1953; Marks et al., 1960; Siegel and Hurst, 1962; Ghul, 1968). This was interpreted as evidence of the birds´ ability to discriminate between familiar and unfamiliar individuals. However, the sharp increase in aggression observed following alteration of the original phenotypes (for all GS) indicates that laying hens responded intensely escalating aggression to the emergence of new phenotypes. Furthermore, this effect was not mitigated by the potential capacity for individual recognition assumed in small groups. This was unexpected considering that the only change applied was to the colour of feathers on the back of the head.

Studies conducted in young meat and laying strains of domestic fowl (Dennis et al., 2008; Campderrich et al., 2017), and other animal species (reviewed by Murray and Fuller, 2000) evidenced that marking can affect health, performance and behaviour, due to social factors or to added difficulties to carry out normal activities. This study explored the process further, showing that similar effects occur not only when a new mark is added (marking 100U), but also when an existing mark is removed (unmarking 100M). To our knowledge, this is the first time this phenomenon has been investigated.

Dennis et al. (2008) proposed four possible mechanisms to explain targeted aggression towards birds with altered phenotypes: 1) fear due to novelty of the marks, 2) xenophobia based on phenotypic dissimilarity, 3) marks perceived as signals of status, and 4) social challenge to conspicuous individuals. Mechanisms 1, 3 and 4 imply that the phenotype of altered individuals is conspicuous as a result of the new dark coloration used. However, our study showed that altered individuals, with or without a dark mark, attracted aggression at similar statistical levels. Thus, our current findings do not support the proposed mechanisms of increased aggression described by mechanisms 3 or 4. We interpret these results as evidence that the change in the phenotypic appearance itself is what caused the escalation in aggression, regardless of GS.

Another possible explanation for our results would involve phenotype matching mechanisms. Phenotype matching is used by animals to learn the phenotypes of their group-mates, creating a template to compare against phenotypes of new, unfamiliar individuals (Mateo and Johnston, 2000; Gerlach and Lysiak, 2006). Phenotype templates are normally shaped by imprinting during the first weeks of age (Bateson 1979; Bateson, 1990; Gerlach and Lysiak, 2006). This short time period is linked to the close proximity of chicks with parents and siblings, which ensures the correct development of phenotype templates. This is essential for survival and fitness, as it will ensure correct species identification for reproduction and recognition of potential competitors for resources. The sudden raise in aggression caused by the PA alteration to homogeneous groups could have been expected in large groups where individual recognition was unlikely (Estevez et al., 1997; Pagel and Dawkins, 1997). However, the lack of GS effects indicated otherwise. PA alteration severely affected aggression even at GS 10, where stable social structures based on individual recognition would have been expected after 33 weeks of cohabitation. These results evidence the birds´ inflexibility to accept new phenotypes once a template has been established. This strong response towards altered phenotypes could be indicating the high impact that ´invaders´ may have had in local populations through their evolutionary history. Invasions of the local populations by unrelated individuals, likely differing in phenotype, may have increased their exposure to new pathogens or parasites (Goodall 1986; Lewis 1998; Hughes and Cremer, 2007) and increased the competition for resources or during mating (Barnard, 1991; Hurst and Barnard, 1992). Aggressively excluding these unrelated phenotypes would have been the most advantageous strategy to ensure the survival of the local population.

Adding a higher proportion of altered birds at T2 (50M/50U, Fig 3.2C) did not have as much impact. Although the level of aggressive interactions was still significantly higher than for control groups, the interactions started declining to reach almost basal levels by T3 (Fig 3.2D). The progressive reduction on aggression as the proportion of altered individuals increased suggests that, despite the social turmoil, birds were able to adapt and incorporate the emerging phenotype into their acceptable templates. Nevertheless, it is important to indicate that in wild populations, a similar escalation in aggression would have been, most likely, sufficient to force birds carrying the new phenotype to leave the group. In this study however, the restriction of the confined environment may have induced the acceptance of the new phenotype over time.

The decline in aggressive interactions could also be explained by a diluting effect (Marin et al., 2014). If birds with altered phenotypes were targeted, then a diluting effect of directed aggression may be occurring as more birds would share the ´cost´ of carrying the new phenotype and fewer original birds will initiate the attacks. It can be argued that, perhaps, a similar decline may have occurred naturally if the groups were left with 30% of altered birds over an extended period of time. This is certainly a likely possibility and the study would have benefited from the inclusion of control pens to test this possibility. However, it was unfeasible to add any further treatments to this large experiment as the facilities were fully occupied.

Despite this shortfall, the strong directionality of the aggressive interactions towards the newer phenotypes revealed by this study is a relevant finding. Aggressive interactions were initiated at a higher than expected rate by individuals from the original and most frequent phenotype, and were clearly directed towards recently altered birds (Fig 3.4B and 3.4C). In 30M/70U groups (controls and recently altered; T1) aggression was directed from U towards M birds, while in 70M/30U (controls and recently altered; T1) the direction was from M towards U birds (Fig 3.4B). A similar pattern was observed in 50M/50U groups at T2, even when proportions of each phenotype within the pens were identical (Fig 3.4C). In addition, lower than expected interactions took place among the most common phenotypes in the groups; UU in 30M/70U (control and recently altered) and MM for 70M/30U (control and recently altered). Previous studies suggested that individuals that look different because of dull or soiled plumage colorations could be considered carriers of transmittable pathogens (Hamilton and Zuk, 1982; Hõrak et al., 2001) and would probably be pushed away from the group. The strong directionality of aggression observed in our study may indicate that unaltered birds were trying to avoid the associated risk of living with unknown phenotypes that could lead to fitness costs. Thus, it is possible that natural factors producing changes in bird appearance, such as injuries, disease or feather pecking, may cause a similar reaction in healthy birds.

The current experiment demonstrates that the directionality of aggression towards altered phenotypes is equally remarkable when emerging as a consequence of adding new dark mark to the feathers, or by removing original dark marks from them. It does appear that the effects of marking were slightly stronger than unmarking birds but differences did not reach statistically significant levels. Even though the frequency of aggressive interactions after introducing phenotypic alterations was high, the large number of treatments may have diluted the potential differences between introducing ´new conspicuous phenotypes´ versus ´new dull phenotypes´. These potential differences should be further explored. However, increased aggression after experimental manipulation of badges of status has been observed in pukekos (*Porphyrio porphyrio melanotus*) (Dey et al., 2014). These results were explained by 'signal incongruence', a mismatch between signal and behaviour, which leads to the animals' attempts at reassessing the accuracy of the signal (Tibbetts and Izzo, 2010). This same mechanism has been argued to explain the despotic behaviour observed towards sick animals (Rohwer and Rohwer, 1978; Goodall, 1986). Birds in our study may have been able to detect a mismatch between their pen mates' appearance and their behaviour. However, altered individuals in each pen were selected at random so a broad representation of social status among altered birds could be assumed. Initial determination of social status was not possible due to the large numbers involved (1050 birds) and the low level of aggressive interactions observed. In any case, both signal incongruence and phenotype matching mechanisms could explain our results as both should produce a similar response increasing aggression towards altered birds.

Despite the low frequency of aggressive interactions observed at T0, directionality was also observed from U towards M birds in 30M/70U control groups (Fig 3.4A). No directionality was detected for 50M/50U or 70M/30U. It could be argued that when phenotypic templates are established at an early age, only the most frequent phenotype would prevail as template for species recognition, by imprinting (Lorenz, 1937) or other mechanisms (Grafen, 1990). In this case, the opposite directionality of aggression would be expected towards unmarked birds in 70M/30U which were no detected at T0. However, after the first PA change at T1, directionality of aggression was observed in control groups from U towards M birds in the case of 50M/50U groups (at T2) and from M towards U in the case of 70M/30U groups (at T1). We speculate that the social instability created by the PA change may have somehow affected the house environment as a whole (i.e. auditory communication), disturbing control pens even if visual contact across treatment pens was not possible. It could be conceivable that under stress even control birds may have increased aggression levels towards individuals with the least frequent phenotypes. Given the age of the birds and the low levels of aggression observed at T0 in all groups, it could be assumed that the social structure, either based in a classic hierarchical system (Schjelderup-Ebbe, 1922; Wood-Gush, 1971; Rushen, 1982) or by the adoption of a tolerant system (Estevez et al., 1997; Estevez et al., 2003), was stable. Despite this, the emergence of new phenotypes clearly triggered a social turmoil with a large increment in the frequency of aggressive interactions. We wrongly predicted increased aggression to be of higher relevance in larger groups. However, GS effects were only observed at T3, when the frequency of aggressive interactions was returning to basal values. Our results provide strong evidences that PA alterations have in fact a much higher relevance than those of GS under the described conditions.

3.6 Conclusion

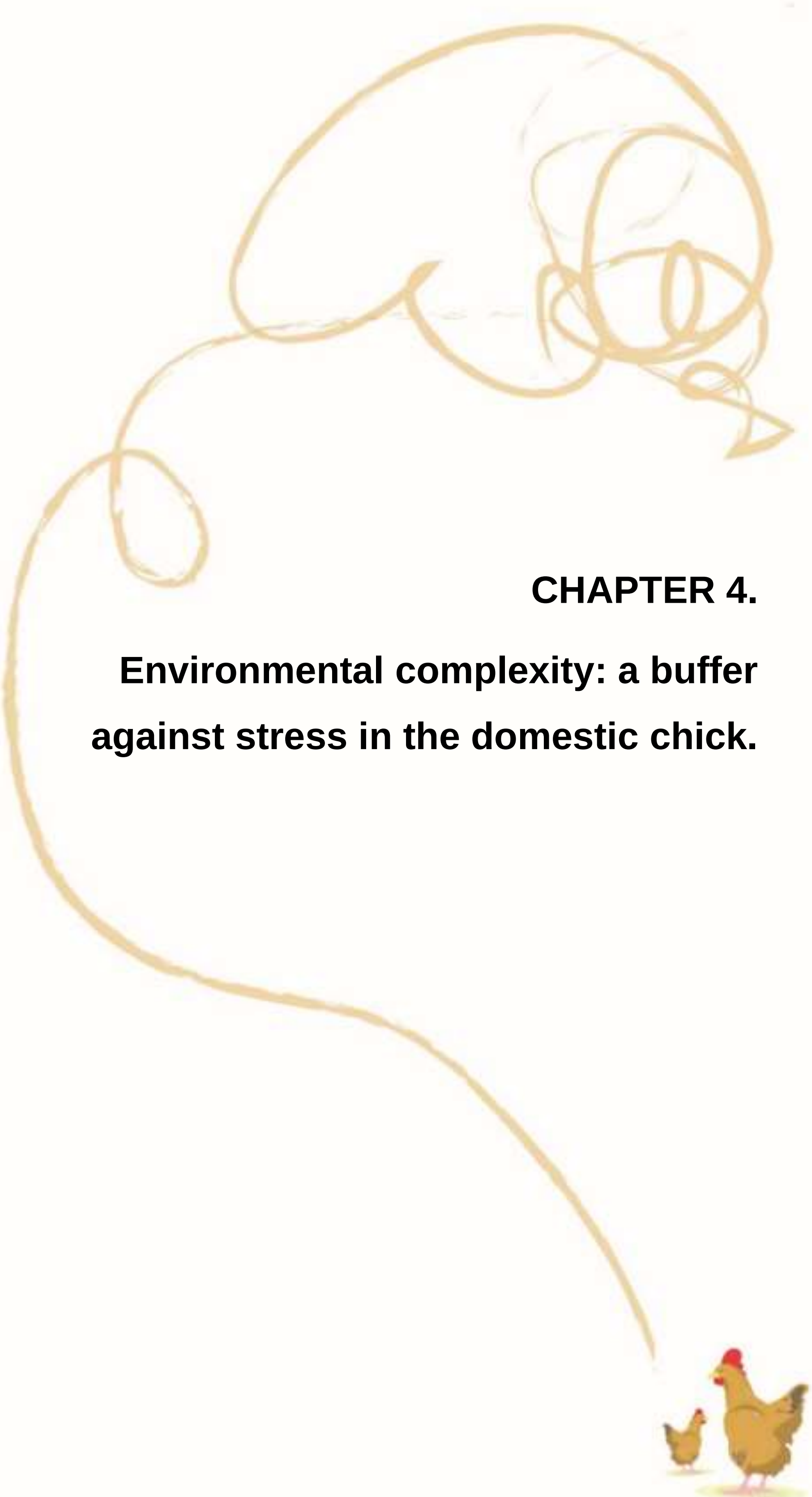
In conclusion, this study provides evidence that the emergence of new phenotypes in originally homogeneous groups of domestic fowl produces an escalation of aggression clearly directed towards birds presenting those new phenotypes. Interestingly, directionality of aggression was equally observed when birds were marked in a homogeneous unmarked population (increasing conspicuosity), and when birds were unmarked in a homogeneous marked population. Therefore, we rejected the status signalling hypothesis as a possible explanation to the effects of phenotype alteration. Phenotype matching mechanisms should be considered as a more parsimonious explanation to the reaction to new phenotypes that we observed. A cost-effective strategy to reduce the risk of competition for resources, or health threats, to the local population from unfamiliar phenotypes should also be factored in. Even though the existence of a phenotypic template could explain the increase on aggression and its clear directionality observed towards altered birds, this may not be a static process. In the case of captive populations, such as farmed birds, the new phenotypes may also be integrated as part of their normal diversity over time, but the process may cause severe stress to the birds until they get acquainted with each other. Only marginal effects of GS were detected, suggesting that the impact of PA was far more important to grant social stability. These findings provide evidence that a simple mechanism, such as phenotype matching, could explain how populations deal and respond to varying phenotypic diversity. Phenotype matching may explain why diversity due to growth, injuries or diseases, could lead to escalation in aggressive interactions that may compromise the survival of the targeted birds.

Acknowledgments

The authors wish to thank I. Gonzalez for her help with animal care and to D. Lizaso and I. Beltrán de Heredia for veterinary assistance.

CHAPTER 4.

Environmental complexity: a buffer against stress in the domestic chick.



4.1 Abstract

Birds kept in commercial production systems can be exposed to multiple stressors from early life and this alters the development of different morphological, immunological and behavioural indicators. We explore the hypothesis that provision of a complex environment during early life, better prepares birds to cope with stressful events as well as buffers them against future unpredictable stressful episodes. In this study, 96 one day old pullets were randomly distributed in eight pens (12 birds/pen). Half of the chicks (N=48) were assigned to a Complex Environment (CENV: with perches, a dark brooder etc.) the others to a Simple Environment (SENV: without enrichment features). Half of the birds from each of these treatments were assigned to a No Stress (NSTR, 33ºC) or to an acute Cold Stress (CSTR, 18-20ºC) treatment during six hours on their second day of life. At four weeks of age, chicks with these four different backgrounds were exposed to an Intermittent Stressful Challenges Protocol (ISCP). In an immunological test indicative of pro-inflammatory status Phytohemagglutinin-P (PHA-P), the response of CSTR birds was ameliorated by rearing chicks in a CENV as they had a similar response to NSTR chicks and a significantly better pro-inflammatory response than those CSTR birds reared in a SENV (five days after the CSTR treatment was applied). A similar better response when coping with new challenges (the ISCP) was observed in birds reared in a CENV compared to those from a SENV. Birds reared in the CENV had a lower heterophil/lymphocyte ratio after the ISCP than birds reared in SENV, independently of whether or not they had been exposed to CSTR early in life. No effects of stress on general behaviour

were detected, however, the provision of a CENV increased resting behaviour, which may have favoured stress recover. Additionally, we found that exposure to cold stress at an early age might have rendered birds more vulnerable to future stressful events. CSTR birds had lower humoral immune responses (sheep red blood cells induced antibodies) after the ISCP and started using elevated structures in the CENV later compared to their NSTR conspecifics. Our study reflects the importance of the early provision of a CENV in commercial conditions to reduce negative stress-related effects. Within the context of the theory of adaptive plasticity, our results suggest that the early experience of the birds had long lasting effects on the modulation of their phenotypes.

4.2 Introduction

Birds used in poultry production systems can be exposed to multiple stressors early in life such as hatching without maternal contact, transport, heat or cold environmental temperature (Appleby et al., 1992; Fraser and Broom, 1997; Lara and Rostagno, 2013). Understanding the impact that these early stressors have on the birds' behaviour and physiology is important from an animal welfare perspective and could, at the same time, elucidate the evolution of phenotypic diversity in populations (Göerlich et al., 2012). In this context, the adaptive plasticity theory proposes that some of the inputs animals receive during their development, perhaps the stressors to which they are submitted or the conditions of their rearing environment, are factors capable of producing lasting alterations. These alterations may, in turn, lead to the development of a certain phenotype that could be adaptive for the individual´s future life. It can, for instance, provide individuals with advantages to face future challenges. On the other hand, it can result in the development of a phenotype with deleterious effects on fitness, perhaps making individuals more sensitive to future stressful conditions (Nettle and Bateson, 2015).

Koolhaas et al. (2011) state that the term 'stress' should be restricted to conditions where the environmental demand exceeds the natural regulatory capacity of an organism. Accordingly, the stressor is defined as a stimulus or environmental condition that induces such a state. This definition of stress was chosen for its simplicity and because it helps in the delimitation of the stress concept. This definition also integrates two aspects of interest for the expression of stress: unpredictability and uncontrollability which will be considered in the development of the present study.

Not all stressors induce the same response and not all individuals cope with stress in the same manner. In fact, among the most important factors determining the stress response are those referring to the individual per se (Dohms and Metz, 1991; Carere et al., 2010) like the birds' genotype, previous experience or health status. Individual differences may explain why a stressor has a profound effect in some birds while having little or no effects in others. Factors such as the nature, intensity, frequency and duration of the stressor also influence its consequences (Dohms and Metz, 1991; Dhabhar and Mcewen, 1997; Muñoz-Abellán et al., 2011) and effects may be different when stressors are applied alone or combined with others (Muñoz-Abellán et al., 2011). When stressors are repeated or are sustained in time, physiological changes may affect diverse body systems. For example, the immune system is influenced by stress response mediators such as corticosterone (Shini and Kaiser, 2009; Shini et al., 2010), whose immunosuppressive effects could reach both to the humoral and cellular components of immunity affecting the efficacy of the responses that characterise this system. Examples of stress induced suppression include; lower relative weights of the thymus and spleen in broilers (Ghazi et al., 2012), a reduction of the Phytohemagglutinin-P (PHA-P) response and of the capacity to produce antibodies against sheep red blood cells (SRBC), together with an elevation of the heterophil/lymphocyte (H/L) ratio in Japanese quail (Nazar and Marín, 2011). A rapid apoptosis in immature T- and B-cells induced by glucocorticoids has also been observed in White Leghorn chickens (Lechner et al., 2001). Stress may have other behavioural (Marin et al., 2001; Cheng et al, 2004) and performance related (Olfati et al., 2018) deleterious effects, which can lead to serious problems or even economic losses for producers (St Pierre et al., 2003; Quinteiro-Filho et al., 2012). At the behavioural level, broilers showed higher fear responses following mechanical restraint than their non-stressed conspecifics (Marín et al., 2001) and white Leghorn chicks were more inactive following an E-coli injection compared to chicks injected with an innocuous saline solution (Cheng et al., 2004). Regarding performance parameters, broilers exposed to heat or cold stress showed reduced feed intake and body weight gain, and an increased feed conversion rate compared to birds reared in thermoneutral conditions (Olfati et al., 2018). These birds also had lower antibody titres against SRBC, indicating a lowered humoral immunological response. Understanding the impact of short and long-term stressors to which young chicks can be exposed in their productive life and exploring the potential to reduce their impact are highly relevant to promote birds´ resilience and thus welfare and performance.

The welfare benefits derived from increasing environmental complexity, for example, by the use of artificial structures such as perches or cover, have been widely documented in poultry (Newberry, 1995; Newberry and Shackleton, 1997; Cornetto and Estevez, 2001a; Cornetto and Estevez, 2001b; Cornetto et al., 2002; Leone et al., 2007; Bizaray et al., 2002; Ventura et al., 2012; Bailie et al., 2013). Providing production animals with opportunities to interact in a complex environment may help them to express more of their natural behaviour (Newberry, 1995) and counteract some stress related deleterious effects (Nazar and Marín, 2011; Benaroya-Milshtein, et al., 2004). The red jungle fowl, ancestor of the domesticated chicken (Wood-Gush, 1959), inhabits forests or areas with vegetative cover (Collias and Collias, 1967) which provides them with opportunities to explore the three-dimensional environment. Moreover, for prey species, the opportunity to take cover or jump onto perches or platforms may be considered a form of controlling their surrounding environment (Keeling, 1997). Such control is probably associated with a positive emotional state of security/safety and it is well documented that controllability helps animals cope better with stress challenges (see Koolhaas et al., (2011), for an extended review).

According to the predictive adaptive response hypothesis, a part of adaptive plasticity theory, the environment that individuals encounter in the early stages of their development will determine their phenotypic plasticity and consequently affect their fitness (Bateson et al., 2014). The humoral and cellular immune responses of quail reared in complex environments were stronger than those from birds reared in barren environments (Nazar and Marín, 2011). Laying hens reared in complex environments also showed better spatial skills than those reared in barren environments (Gunnarsson et al., 2000) and thus could be expected to adapt better to new environments. Taking these into consideration, we propose that providing birds with a complex environment early in their development will increase their plasticity at the immunological and behavioural level, thus better preparing them to confront the challenges they may encounter later in life. We explore the hypothesis that provision of a complex environment during early life prepares birds to cope better with stressful events or buffers them from future unpredictable stressful episodes. Additionally, we investigate whether controlled exposure to early stress increases birds´ resilience or whether it actually amplifies their sensitivity to future challenges.

In the present study, we worked with an integrated model that allowed us to identify the consequences of acute and unpredictable stressful challenges in domestic chicks reared in simple or complex environments (SENV or CENV respectively). We predicted that experiencing a CENV would attenuate the impact of acute stress and improve birds’ ability to cope with future unpredictable challenges. If these predictions are confirmed, it would be possible to develop protocols for the improvement of birds’ resilience to stress, thus having a positive impact on their welfare and performance.

4.3 Material and methods

4.3.1 Animals, transport and housing conditions

One hundred and four domestic fowl chicks (*Bovan robust*, a white layer strain) were purchased from a Swedish hatchery (Swedfarm AB, Linköping) in June 2014. The chicks were collected the day of hatching and immediately transported to our experimental facilities at Lövsta (Swedish University of Agricultural Sciences). The experimental room contained eight (1.2 x 1.2 m) pens built with a wooden structure and wire mesh. Visual contact across neighbouring pens was avoided by placing 97 cm high wooden barriers between the pens. The birds were fed *ad libitum* with a standard commercial diet, designed according to their rearing phase. Feed and water were provided in round shaped feeders (12 cm/ pullet) and bell drinkers (four cm/pullet) and wood shavings were used as litter. The room had automatically controlled photoperiods, temperature and ventilation that were adjusted during the rearing according to the recommended commercial practices for pullets (Bovans Robust-Swedfarm, 2014).

Upon arrival, all chicks were individually weighed (Sartorius BL 1500 measuring to the nearest 0.1 g) and white numbered leg rings were placed on both legs for individual identification. Birds were ranked on day two according to body weights and were distributed across eight groups of 13 chicks in a balanced manner to provide similar mean weights for each group. Even though the whole study was performed with 96 pullets and experimental final groups consisted of 12 birds, we placed an extra chick in each group for the first week to compensate for possible early mortalities. After that time, in groups where no deaths occurred, the extra chick was removed and placed in an extra pen in the same room. These birds were used in additional pilot studies and then at the end of the third week they were given away to private homes.

At eight days of age birds were tagged with two, 3 cm diameter, white laminated round-shaped paper tags attached by a thin plastic filament through the skin of each wing (Cornetto and Estevez 2001a). The tags had numbers printed on both sides to facilitate visual individual identification. Leg rings were removed at three weeks of age.

4.3.2 Ethical note

This project was performed according to the ethical requirements regarding animals used for scientific purposes established in Sweden. The experimental protocol was approved by the Uppsala Ethical Committee (ethical permit number C70/14). The humane endpoints used in this study were any bird losing more than 10% of its bodyweight, having any large injury or being sick and unable to stand normally. Birds were checked twice per day and weighed each week. Birds reaching any of these endpoints were euthanized. Euthanasia was by dislocation of the neck, which was preceded by stunning. In addition to the pre-determined endpoints, during the study birds showing any type of sickness behaviour were monitored regularly throughout the day and their situation discussed with the responsible veterinarian. If birds did not start to show signs of recovery they were euthanized. Birds that started eating, drinking and moving around the pen were considered to be recovering.

4.3.3 Experimental design

Cold stress (CSTR) was used in this experiment as an acute environmental stressor to simulate a stressful situation that chicks may experience during transport from the hatchery to the rearing facilities. It is known that thermoregulation in chicks is underdeveloped the first ten days after hatching (Baarendse et al., 2007), which makes them especially vulnerable to cold stress (Widowski, 2010). Birds from stressed (CSTR) and non-stressed (NSTR) groups were reared in complex and simple environments (CENV and SENV, respectively) to examine the potential short and long-term buffering effects of the complex environment on birds´ morphology, immunology and behaviour. In addition, birds were exposed to an Intermittent Stressful Challenges Protocol (ISCP) with the goal of assessing birds’ coping ability according to their early life experiences. The ISCP consisted of exposure to stressors applied at different hours and different days during a week. Uncontrollable and unpredictable events have been described as major sources of stress in rodents (reviewed in Koolhaas et al., 2011) and unpredictability may also significantly affect learning abilities and long-term behaviour in domestic fowl (Lindqvist et al., 2007; Ericsson et al., 2016).

A two-factorial design combining the effects of stress and environmental complexity was used. Half of the birds (48) were assigned to either a SENV or a CENV treatment. The SENV housing contained wood shavings, a feeder and a drinker. The CENV had the same features but, in addition, the birds had access to small solid blocks (10 x 25 x 5 cm; W x L x H), round perches (110 cm x 2.5 cm; L x diameter) at four levels (15, 32, 52 and 66 cm; H) and a hide area (40 x 110 x 20 cm; W x L x H). All the structures were constructed of wood. The hide could be used as a dark area to rest in and the roof could be a platform to roost on. See Fig 4.1 for a detailed description of the SENV and CENV treatments.

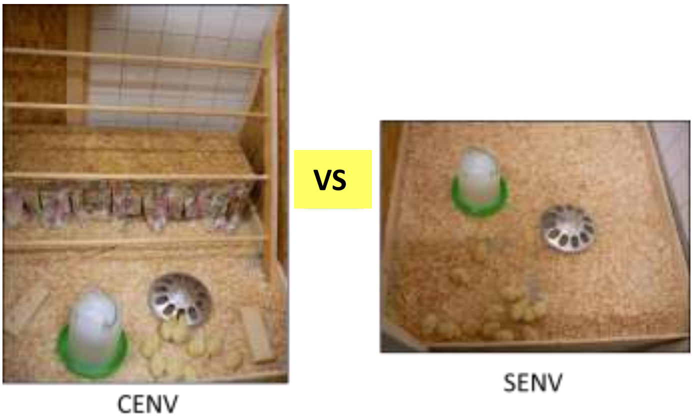


Figure 4.1 *Complex versus Simple environments. Photo of two pens from the current experiment; the one on the left side represents the Complex Environment (CENV) treatment provided with a drinker, a feeder, wood shavings, three wooden blocks, four perches at different levels and a hide area; while the one on the right side represents the Simple Environment (SENV) treatment, provided only with a drinker, feeder and wood shavings.*

On day two, half of the chicks from each environment were assigned to a CSTR treatment while the other half were allocated to the NSTR treatment. For this, all chicks from each pen were collected and were placed in pairs in a compartmented cardboard box (box size 40 x 60 x 15 cm; compartment size 20 x 15 x 15cm; W x L x H). Boxes with CSTR birds were kept in a room under suboptimal temperatures of 18-20ºC during six hours. NSTR boxes were kept at a standard temperature of 33ºC. To ensure chick welfare during the treatment exposure and to verify that chicks were indeed CSTR, we monitored rectal temperatures with a digital thermometer (DocMorris CE 0123) introduced one cm into chicks´ cloaca every two hours. After the stipulated six hours, birds were returned to their home pens. The locations of the pens with birds allocated to the environmental (CENV and SENV) and stress (CSTR or NSTR) treatments were balanced in the room and split into two blocks (See Fig 4.2A and 4.2B for a detailed description of the experimental set up and distribution of the experimental groups).

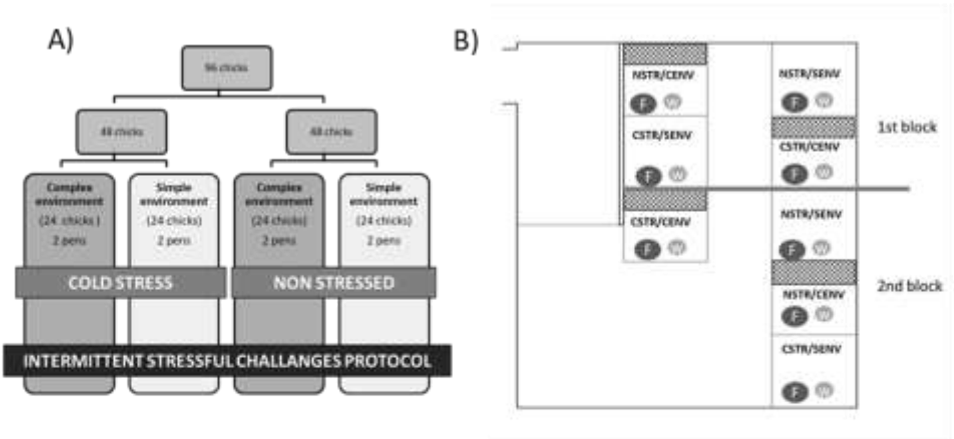


Figure 4.2 *Experiment description. (A) Experimental design: for this experiment 96 domestic fowl chicks (Bovan robust, a white laying strain) were randomly assigned to either a Complex or a Simple home environment (CENV, SENV respectively; N=48 per treatment). Additionally, birds from each environment were allocated to either a Cold Stress (CSTR) or No Stress (NSTR) treatment. At four weeks of age birds with different early life experiences (CSTR-CENV, CSTR-SENV, NSTR-CENV, NSTR-SENV, two pens/treatment, eight pens in total) were exposed to an Intermittent Stressful Challenges Protocol (ISCP); (B) Distribution of the treatments across the experimental room: each square division represents a pen, F indicates the feeder, W the drinker, CENV refers to Complex Environment, SENV to Simple Environment; NSTR refers to a No Stress group while CSTR refers to a Cold Stress group.*

To test how pullets with different early life experiences cope with new challenges, they were exposed to an Intermittent Stressful Challenges Protocol (ISCP) at four weeks of age. The ISCP used in the present study was applied during five consecutive days, from 23 to 27 days of age, and multiple uncontrollable and unpredictable stressors applied to the entire room were used. The stressors consisted of (1) random normal commercial practices, such as modification to the heating system (day 23 of age), changing the feeders and drinkers for bigger ones and cleaning the pens, which included removing and replacing the bedding material (day 27 of age); (2) one hour exposure to heavy metal music played at 90dB, all five days in a row at different times of the day; (3) daily exposure to unpredictable loud noises (reproduced also at 90dB), which included recorded sounds of predators or other animals and mechanical sounds of trains, airplanes or ambulances, and (4) changes in the timing of the light/dark phases by programming the lights to switch on and off at random intervals during the last three days (day 25 to 27 of age).

In order to explore the reaction of the pullets to the ISCP, every second day during the exposure to heavy metal music, we registered every 15 min the number of chicks that were feeding. We started our observations 30 min before the music was switched on and stopped 30 min after it was switched off.

4.3.4 Dead birds

Only three birds from three different treatments had died by the end of the first week. The extra chicks in those groups were used to complete the experimental group, while extra chicks in other groups were removed and placed in an extra pen as described previously. No additional mortalities were observed until the sheep red blood cells (SRBC) injection at four weeks of age. The first deaths were detected during the morning check the day after the injection (12 hours after injection). Bacterial contamination of the injected blood is a possible explanation for these mortalities, but we cannot confirm this. A total of 24 chicks either died (n=11) or were euthanized (n=13). The mortalities were an unfortunate and unexpected event following this routine challenge procedure. Some chicks were euthanized immediately, some sick birds recovered and the time interval between the injection until euthanizing the last of the non-recovering birds was 2 days. Criteria for the decision of when to euthanize is described in the ethical note section. The mortalities are shown in detail in Table 4.1. Since there was no longer any possibility to replace birds, all subsequent statistical analyses were corrected for the different numbers of birds in the pens. The mortalities resulted in differences in stocking density and resource allocation between pens for the remainder of the study.

Table 4.1 *Dead and euthanized birds after the injection of SRBC. The first column indicates the pen number; the second column indicates the corresponding treatment (CSTR/NSTR referring to the Cold Stress or No Stress treatments and SENV/CENV referring to the Simple or Complex Environments respectively); the last column lists the identity numbers of the birds in the pen; the birds that died are marked in yellow and those that had to be euthanized are marked in light grey.*

Pen Nº	Treatment	Birds Id											
1	CSTR/CENV	84	80	71	81	77	60	22	5	75	46	58	8
2	CSTR/SENV	89	28	38	24	37	101	54	65	25	12	4	6
3	NSTR/CENV	41	95	88	49	44	76	32	18	13	50	78	48
4	NSTR/SENV	97	26	31	43	2	90	42	27	14	85	91	96
5	CSTR/CENV	45	59	21	70	29	10	61	57	82	53	63	79
6	NSTR/SENV	20	86	68	83	30	35	3	73	64	7	51	15
7	NSTR/CENV	72	69	92	23	74	39	102	87	52	47	17	9
8	CSTR/SENV	66	16	55	33	94	19	34	36	56	1	11	93

4.3.5 Morphological measures

All birds were individually weighed (Sartorius BL 1500 measuring to the nearest 0.1 g) once per week during the experiment (weeks one to six). The increase in grams per week was used to calculate growth rate. At the end of the experiment (week six), tarsus length and width were measured following the procedure described by Campo and Prieto (2010) with a digital calliper (Biltema, Sweden) to the nearest 0.01 mm. Body weight and tarsus measures were analysed separately but also combined to calculate body condition (calculated as the ratio: body weight/tarsus length). Relative fluctuating asymmetry (RFA) was calculated as the absolute difference between the right and left legs divided by the mean between the left and right measures

(Møller et al., 1995; Yngvesson and Keeling, 2001). These morphological indicators were selected because of their previously documented association to measures of individual quality, health status and stress levels (Møller et al., 1995; Yngvesson and Keeling 2001; Quinteiro-Filho et al., 2012; García-Navas et al., 2013; Rodríguez et al., 2016; Olfati et al, 2018).

4.3.6 Immunological variables

Based on previous studies (Nazar et al., 2015) the following variables were used to assess humoral and cellular immune responses at different experimental stages; (1) heterophil/lymphocyte (H/L) ratio (Nazar and Marín, 2011; Huff et al., 2005; Nelson et al., 2018) was used as a cellular representative, as well as a haematological indicator of chronic stress. Elevated H/L ratios have previously been associated with high stress levels (Campo et al., 2008; Nelson et al., 2018); (2) the lymphoproliferative response to phytohemagglutinin-p (PHA-P) (Roberts et al., 2009; Vinkler and Bainova, 2010; Nazar and Marín, 2011) was also used as a cellular representative and as an indicator of the pro-inflammatory potential of each bird; and (3) the primary antibody response against sheep red blood cells (SRBC) (Smits and Baos 2005; Adriaansen-Tennekes et al., 2009; Nazar and Marín, 2011) was used as a humoral indicator of acquired immunity. The procedure and time schedule for each immunological test is summarised in Table 4.2. Analyses of the physiological measures were carried out blind to the treatment.

Table 4.2 *Immune variables measured and time schedule for each test performed.* T1, T2, T3 and T4 are times 1-4 respectively; CSTR/NSTR are the Cold Stress and No Stress treatments; ISCP refers the Intermittent Stressful Challenges Protocol.

Variable measured	Procedure	Sampling Time
Heterophil/ lymphocyte (H/L) ratio	The left-wing brachial vein of each bird was punctured to obtain one blood drop for smears. Leukocyte counts were obtained by analysing the blood smears stained with May Grünwald Giemsa using an optic microscope. The proportion of each leucocyte type was calculated over counts of 100 white cells per smear. Afterwards, the H/L ratio was calculated for each bird and sampling day.	1st smear collection: Day 3 (T1), one day post exposure to CS/NS treatment.
		2nd smear collection: Day 18 (T2), before exposure to ISCP.
		3rd smear collection: Day 28 (T3), post ISCP exposure.
		4th smear collection: Day 36 (T4) one week after ICSP exposure.
Lymphoproliferative response to phytohemagglutinin-p (PHA-P) injection	Each bird was injected with 0.05 ml of a PHA-P solution in phosphate saline buffer (1mg/ml) in the wing web (intradermal injection). The injection site was marked with a non-toxic permanent marker. Wing web thickness was measured just before and 24h after the PHA-P injection. The dermal swelling response was reported as the percentage increase in wing web thickness at the injection site. Measurements were recorded using a digital calliper (Biltema, Sweden) to the nearest 0.01 mm.	1st Injection: On Day 7, five days post exposure to the CSTR/NSTR treatment. Inflammation was determined 24 h later.
		2nd Injection: On Day 28, after birds were exposed to the ISCP. Inflammation was determined 24 h later.
Primary antibody response against sheep red blood cells (SRBC)	Birds were intraperitoneally injected with 0.05 ml of a 10% solution of (SRBC) to induce a humoral immune response. One week after the SRBC administration, 0.5 ml of blood from each bird was collected from the brachial vein opposite to the wing used in the PHA-P test. Syringes were filled with two drops of E.D.T.A K3 to avoid coagulation. Blood samples were kept in Eppendorf tubes placed on ice till centrifuged. Samples were centrifuged at 2500 rpm/15 min and the serum obtained was distributed in Eppendorf tubes and kept at -20ºC until analysis. The antibody response was assessed with a microagglutination assay (Server, 1961; Smits and Williams, 1999; Smits and Baos, 2005; Adriaansen-Tennekes et al., 2009).	Injection: On Day 28, after birds were exposed to the ISCP.
		*Due to a possible contamination of the injected blood, a total of 24 chicks either died or were euthanized. Data were corrected to the actual group size to account for this.

4.3.7 Behaviour and use of space in home pens

Direct behavioural observations according to a standardised ethogram (Table 4.3, adapted from (Newberry et al., 2007; Liste et al., 2015) were conducted two times per week between 9:00-15:00h. The exception to this was week four, when the ISCP was applied, when observations were performed only once. A scan sampling approach was used in which the observer noted the number of birds performing each behaviour at the time of the scan (Arnould and Faure, 2003; Liste et al., 2015). The general activities included; resting, standing, walking, preening, eating, foraging and drinking.

For birds in the CENV, we also noted their locations (on a perch, in the hide area, on the hide area platform, on a wooden block or on the floor) at the time of the scan. When a bird was observed perching, the perch level was also registered. A total of 20 scans/day were conducted across the eight experimental pens, divided into two morning and two afternoon blocks. Each block therefore consisted of five scans and these were performed in a random order across the eight pens. Since the pens looked different, the person doing the observations could not be blind to the environmental treatment. For a better overview, a detailed chronogram of all the treatments and tests performed during the study is shown in Fig 4.3.

Table 4.3 *Ethogram used for the data collection.*

Behaviour	Description
Eating	Pecking and/or eating at feeder.
Drinking	Pecking and/or drinking at drinker.
Foraging	Pecking and scratching in litter.
Resting	Lying down inactive or standing with closed eyes or head tucked under wing.
Standing	Standing inactive with open eyes (includes both alert and non-alert states).
Walking	Bird takes ≥2 steps forward.
Preening	Bird arranges or oils her feathers with her beak.

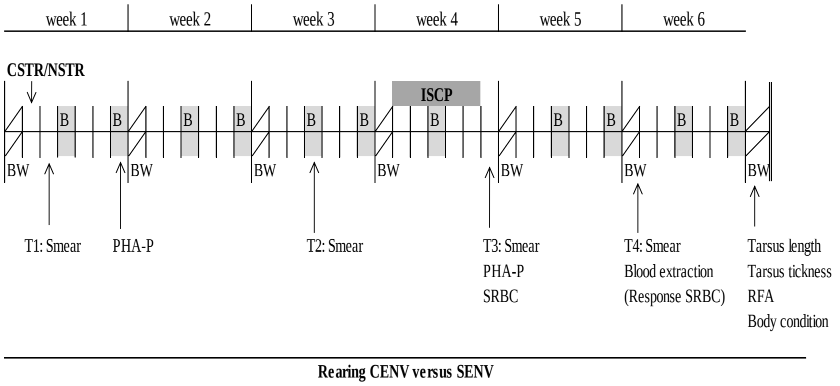


Figure 4.3 *Chronogram of the study. Body weight (BW) was measured the first day of the study in order to balance groups accordingly and every week afterwards; birds were either assigned to a Complex Environment (CENV) or to a Simple Environment (SENV) as their rearing system; on day two of age, half of the birds from each environment were submitted to either a Cold Stress (CSTR) or a No Stress (NSTR) treatment; behavioural observations (B) were performed two times per week except for week four when the Intermittent Stressful Challenges Protocol (ISCP) was applied. The time of the measure of each immunological parameter (smears for accounting the heterophil/lymphocyte ratio (H/L ratio), Phytohemagglutinin-P injection (PHA-P) and Sheep Red Blood Cells injection (SRBC)) are indicated by arrows in the chronogram; at the end of the study we also collected tarsus weight, tarsus thickness and calculated body condition and Relative Fluctuating Asymmetry (RFA).*

4.3.8 Statistical analyses

The GLIMMIX procedure in SAS V.9.3 (SAS Inst. Inc., Cary, NC) was used for the analysis of data in this experiment. Different distributions were used in the different analyses depending on the most appropriate fit for the data as detailed below. A compound symmetry matrix was used for those analyses with repeated measures over time. When necessary a Konward-Roger adjustment for the degrees of freedom was used (Littell et al., 2006). The details of the specific analyses are given below.

4.3.8.1 Effect of cold stress/no stress treatment on internal body temperature

Firstly, we explored whether the CSTR/NSTR treatment affected the internal body temperature of the birds during the six hours of exposure. CSTR/NSTR treatment, time of exposure (0, 2, 4, 6 hours) and their interaction were included in the model as fixed effects. Time of exposure was included as a repeated measure and bird identity nested to pen as the subject. Data fitted into a gamma distribution. To validate that this was an acute stressor, the internal body temperature of the cold stressed chicks should be significantly lower than that of the non-stressed chicks and the absolute environmental temperature used should be at least that previously shown to be associated with cold stress effects on behaviour (Mujahid and Furuse, 2009).

4.3.8.2 Effect of intermittent stressful challenges protocol on feeding behaviour

To investigate the effect of at least one of the stimuli on the birds, the effect of the repeated exposure to one hour of heavy metal music reproduced at 90dB on the number of birds feeding was explored. In this case CSTR or NSTR treatment, CENV or SENV treatment, day and time of exposure (30 and 15 min before music reproduction (no music), 0, 15, 30, 45, 60 min (during music reproduction) and after music was switched off at 75 and 90 min (no music)), were included in the model as fixed effects. Day was introduced as random and pen as subject. Data fitted a normal distribution after square root transformation.

4.3.8.3 Morphological and immunological indicators

For those variables that were taken only once during the experiment (tarsus length, tarsus thickness, body condition, RFA and antibody response to SRBC injection) we used a model that included CSTR/NSTR treatment, CENV/SENV treatment and their interaction as fixed effects. Pen was included as a random effect and bird identity nested to pen as the subject. For those variables which were taken repeatedly during the experiment (body weight, swelling response after PHA-P injection, H/L ratio) the model included CSTR/NSTR treatment, CENV/SENV treatment, age and their interactions as fixed effects. Age was introduced as repeated measure and bird id nested to pen as the subject. Variables fitting a normal distribution were (1) raw data from the tarsus thickness, (2) square root transformed data from the RFA (tarsus length and thickness) and the swelling response after PHA-P injection; (3) log2 transformed data from the antibody response to the injection of SRBC. Body weight, body condition and tarsus length fitted a gamma distribution and H/L ratio fitted a lognormal distribution.

4.3.8.4 Behavioural indicators

Regarding behavioural activity, the mean frequency of pullets performing each of the mutually exclusive behaviours was averaged first by observation round (mean of five scans/pen), and then by day and week. Data were corrected for the actual group size per pen at the time of the scan. Eating, drinking, foraging, preening, resting, standing and walking frequency fitted a normal distribution. CSTR/NSTR treatment, CENV/SENV treatment, week of age and their interactions were included in the model as fixed effects. Week of age was included as a repeated measure and pen as the subject.

4.3.8.5 Use of space

For the CENV treatment the mean frequency of birds on the floor or using the structures provided (perches, hide area, platform over the hide area or the wooden blocks) was calculated per scan and data were corrected for group size. The mean frequency of birds using each structure by observation round (mean of five scans/pen) day and week was calculated. Data for the use of the hide area and the floor followed a normal distribution, while data from the use of the perches, wooden blocks and the platform on the hide area were square root transformed. CSTR/NSTR, week of age and their interactions were included in the models as fixed effects. Week of age was introduced as a repeated measure and pen as the subject. Additionally, potential effects of CSTR/NSTR and week of age on the use of the different levels of the perches (1, 2, 3, 4) were explored. In this case we calculated the mean frequency of birds using each perch level per observation round (mean of five scans/pen corrected by group size) day and week. In this analysis we included CSTR/NSTR, week of age and perch level as fixed factors, as well as, CSTR/NSTR by perch level and week of age by perch level interactions. Week of age was introduced as a repeated measure effect and pen as a subject. Data were fitted to a normal distribution after square root transformation.

4.4 Results

4.4.1 Effect of acute cold stress on internal body temperature

There was a significant effect of the CSTR/NSTR treatment, time and their interaction (CSTR/NSTR treatment x time) on chicks' body temperature ($F_{1,102}$=63.3, p<0.0001; $F_{3,304}$=263.26, p<0.0001; $F_{3,304}$=94.64, p<0.0001 respectively). Body temperature decreased in both stressed and non-stressed birds. However, as shown in Fig 4.4, body temperature in CSTR birds decreased significantly more than in NSTR birds (average decrease of 0.8 ºC for NSTR vs. 2.9 ºC for CSTR).

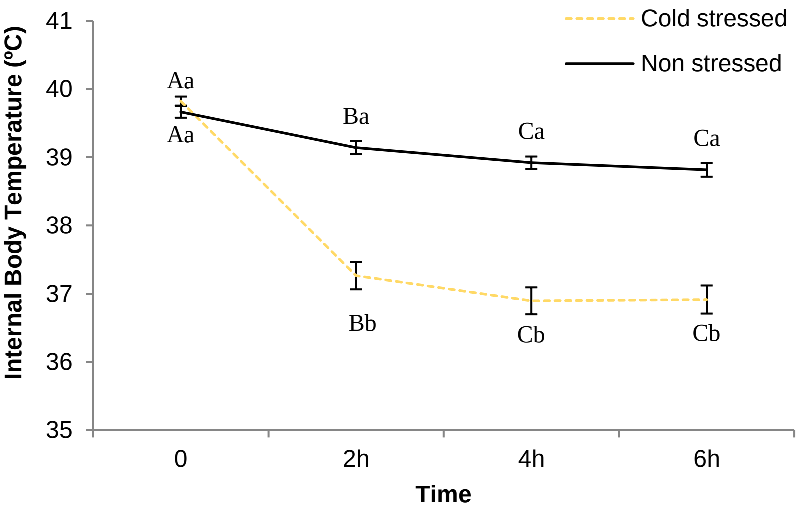


Figure 4.4 *Internal body temperature for Cold Stress (CSTR) No Stress chicks (NSTR). Mean (± SE) internal body temperature for acute CSTR and NSTR chicks; temperature was registered in ºC every 2h, at 0 (beginning of the cold stress), 2h, 4h, and 6h;* $^{(A\text{-}C)}$ *letters indicate differences over time within the CSTR or NSTR treatment;* $^{(a\text{-}b)}$ *letters indicate differences between CSTR and NSTR birds at the same time* point.

4.4.2 Effect of the intermittent stressful challenges protocol on feeding patterns

There was an effect of time ($F_{8,56}$=17.16, p<0.0001) on feeding activity during the one-hour exposure to heavy metal music in the ISCP. Fig 4.5 illustrates the differences across time on feeding activity. Birds drastically reduced their feeding activity when the music started. Feeding activity increased in the final 15 minutes of music exposure and returned to pre music levels after the music was switched off. No differences across days, CENV/SENV or CSTR/NSTR treatments were found (p>0.05).

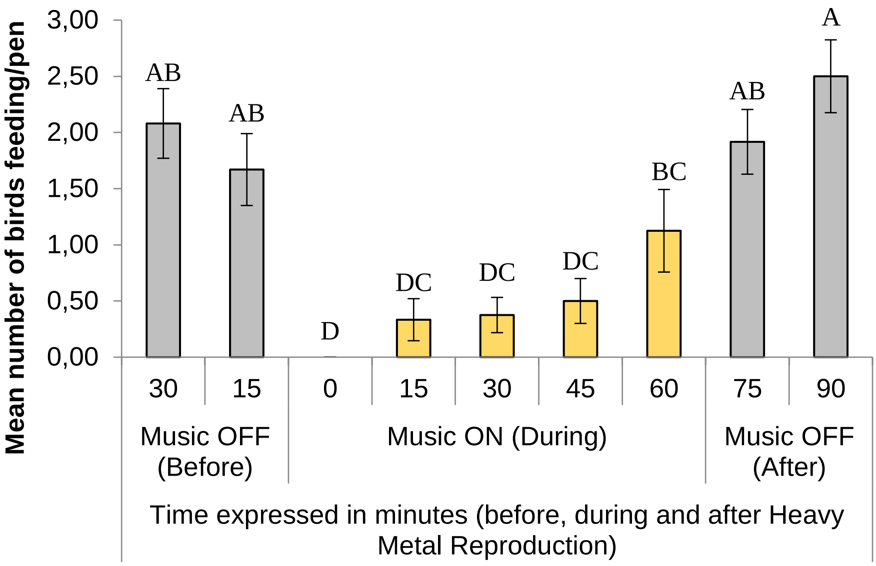


Figure 4.5 *Number of animals feeding during application of the intermittent stressful challenges protocol (ISCP). Mean (± SE) number of animals feeding before (30, 15 min), during (0, 15, 45 and 60 min) and after (75 and 90 min) exposure to heavy metal music reproduced at 90Db; $^{(A-D)}$ letters indicate differences across time.*

4.4.3 Body growth and other morphological indicators

An effect of CENV/SENV treatment, age and their interaction was found when analysing body growth between consecutive weeks ($F_{1,92.64}$=6.43, p=0.01; $F_{4,410.2}$=805.54, p<0.0001; $F_{5,410.2}$=2.30, p=0.04 respectively). Chicks reared in a CENV showed a slightly slower growth curve compared to those from SENV for age periods 3, 4 and 5 (Fig 4.6). However, birds from a CENV seem to compensate by growing faster and reached similar levels to SENV birds by the end of the study. No effects of the CSTR/NSTR treatment were detected for this variable (p>0.05).

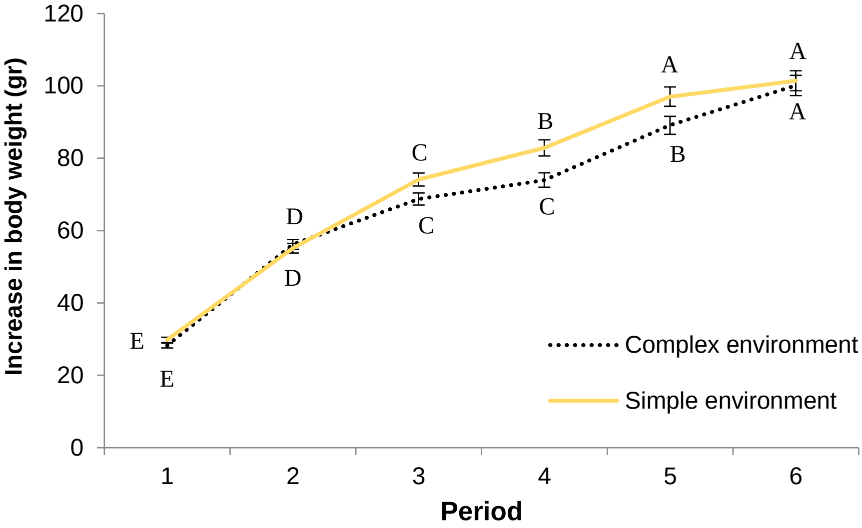


Figure 4.6 *Increase in body weight estimated between two consecutive weeks. ^(A-E)^ letters indicate significant differences across time within each CENV or SENV treatment (p<0.05); there were no significant differences between CENV/SENV treatments at the same age period.*

At the age of six weeks, pullets reared in the CENV had a shorter tarsus and a lower body condition than those reared in the SENV (tarsus length; CENV: 59.01±0.42, SENV: 60.30±0.41, $F_{1,68}$=5.08 p=0.03; body condition (body weight/tarsus length); CENV: 7.64±0.07, SENV:7.87±0.07, $F_{1,68}$=4.49, p=0.04). No differences in tarsus thickness ($F_{1,68}$=0.06, p=0.81) or in RFA for tarsus thickness ($F_{1,69}$=0.34, p=0.56) were detected according to the complexity of the rearing environment. However, there was a tendency for a smaller RFA for tarsus length in the simple environment (CENV: 0.02±0.01, SENV: 0.01±0.009, $F_{1,68}$=3.02, p=0.09). No effect of CSTR/NSTR treatment or the CSTR/NSTR by CENV/SENV interaction were found for any morphological parameter (p>0.1).

4.4.4 Immunological indicators

4.4.4.1 Heterophil/lymphocyte ratio

The results of the H/L ratio are reported in Table 4.4 and reveal a significant effect of the three-way interaction CSTR/NSTR by CENV/SENV and age (Fig 4.7). In this case, CSTR birds showed a higher H/L ratio than NSTR at day three of age (T1), directly after exposure to CSTR/NSTR treatment, suggesting high stress (Campo et al., 2008; Nelson et al., 2018). By T2, the H/L ratio in CSTR birds had decreased to levels similar to that of the NSTR birds. No effect of the rearing environment was observed in either of these first two sampling points.

Table 4.4 *Results from the statistical analyses regarding heterophil/lymphocyte (H/L) ratio and the swelling response after the phytohemagglutinin-P (PHA-P) injection, expressed as percentage of inflammation.*

	H/L Ratio			**Percentage of Inflammation**		
Effects	**F-value**	**DF**	**p-value**	**F-value**	**DF**	**p-value**
CSTR/NSTR	78.87	1,96.15	<0.0001	20.53	1, 73.32	<0.0001
CENV/SENV	115.11	1,96.15	<0.0001	15.32	1, 73.32	0.0002
Age	93.39	1,253.8	<0.0001	249.22	1, 80.55	<0.0001
CSTR/NSTR x CENV/SENV	6.67	1,96.15	0.01	39.95	1, 73.32	<0.0001
CSTR/NSTR x Age	34.24	1,253.8	<0.0001	12.25	1, 80.55	0.0008
CENV/SENV x Age	47.08	1,253.8	<0.0001	0.11	1, 80.55	0.74
CSTR/NSTR x CENV/SENV x Age	2.59	1,253.8	0.05	9.70	1, 80.55	0.003

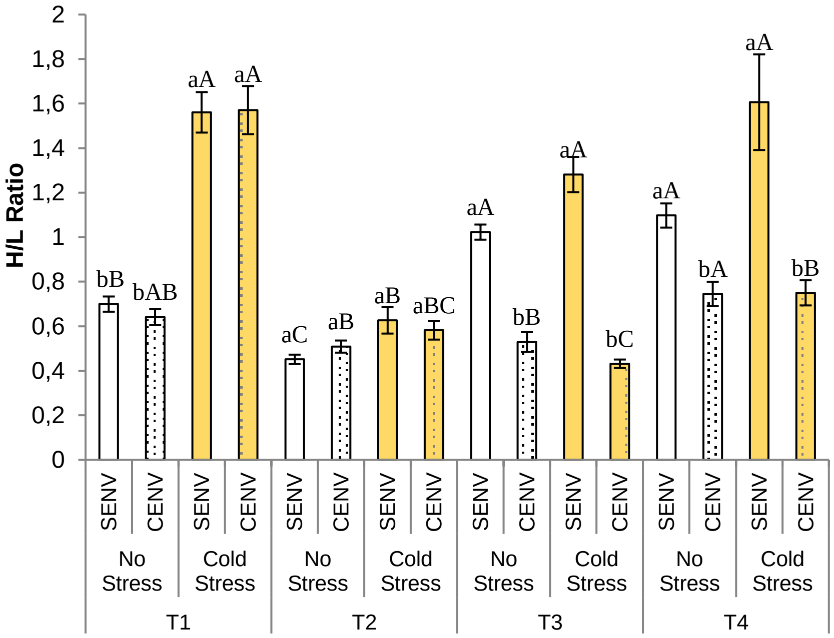


Figure 4.7 *Heterophil/lymphocyte ratio evolution according to the different treatments. Mean (± se) Heterophil/lymphocyte (H/L) ratios in Cold Stress (CSTR) and No Stress (NSTR) birds kept in the Simple Environment (SENV) and a Complex Environment (CENV); NSTR treatment is represented by white bars, while CSTR by yellow bars; SENV is represented by bars with a solid colour and CENV by bars with dots; ${}^{(a\text{-}b)}$ letters indicate differences between treatments at each sampling day and ${}^{(A\text{-}C)}$ letters indicate differences between the four different sample points; (T1) 2 days after hatching (directly after the exposure to the CSTR/NSTR treatment); (T2) 18 days after hatching; (T3) 28 days after hatching (just after the 5-days of exposure to the ISCP); (T4) 36 days after hatching (one week after the exposure to the ISCP).*

Interestingly at T3, directly after exposure to the ISCP, birds kept in the SENV treatment had a significantly raised H/L ratio irrespective of whether they had been submitted to the CSTR or to the NSTR treatments early in life (Fig 4.7). This increase persisted and at T4 birds from the SENV had higher H/L ratios compared to birds in the CENV.

4.4.4.2 Lymphoproliferation to PHA-P injection and antibody response to SRBC

Results from the swelling response after PHA-P injection are reported in Table 4.4 Similar to the results for the H/L ratio, the results revealed a significant effect of the triple interaction CSTR/NSTR by CENV/SENV and age (Fig 4.8). Birds that were CSTR and reared in a SENV showed the lowest swelling response after the first PHA-P injection, some days after the exposure to the CSTR/NSTR treatment and immediately after the ISCP was applied. All groups were found to have significantly diminished inflammatory response after being submitted to the ISCP.

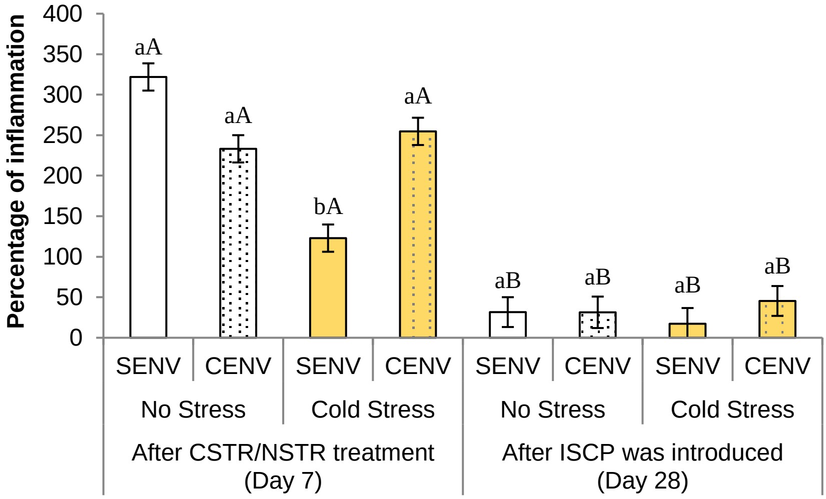


Figure 4.8 *Mean (± SE) percentage of inflammation measured after PHA-P injection.* $^{(a\text{-}b)}$ *letters indicate differences between different treatments at each injection time i.e. Day 7 of age (five days after Cold Stress (CSTR)/No Stress (NSTR) treatment) and Day 28 of age (after the ISCP was applied);* $^{(A\text{-}B)}$ *letters indicate differences across time within the same treatment; NSTR treatment is represented by white bars; CSTR by yellow bars; SENV is represented by bars with a solid colour; CENV by bars with dots.*

An effect of CSTR/NSTR treatment was found when analysing the antibody response to SRBC ($F_{1,57}$=5.03, p=0.03). CSTR birds had a significantly lower production of antibodies than NSTR birds (CSTR: 2.40±0.32, NSTR: 3.38±0.32). No effect of CENV/SENV or of the interaction was found (p>0.05).

4.4.5 Behaviour in the home pen

Chicks in the CENV rested more, as well as they stood and walked less than chicks in the SENV (resting: $F_{1,5}$=17.67, p=0.008, CENV: 0.43±0.01 SENV:0.34±0.01; standing: $F_{1,5}$=8.12, p=0.04, CENV: 0.1±0.005, SENV:0.13±0.007; walking: $F_{1,5}$=19.62, p=0.007, CENV: 0.09±0.004, SENV: 0.12±0.004). No effect of CENV/SENV was detected for any of the other behaviours (preening, eating, foraging and drinking, p>0.05 in all cases). No effect of the CSTR/NSTR treatment was observed for any of the behaviours studied. An effect of week of age was found for eating ($F_{5,35}$=6.70, p=0.0002), foraging ($F_{5,35}$=13.73, p<0.0001), preening ($F_{5,35}$=11.60, p<0.0001), standing ($F_{5,35}$=6.87, p=0.0001), walking ($F_{5,35}$=6.00, p=0.0004) and drinking ($F_{5,35}$=6.30, p<0.0003), (Fig 4.9). No significant differences on resting were observed across time (p>0.05).

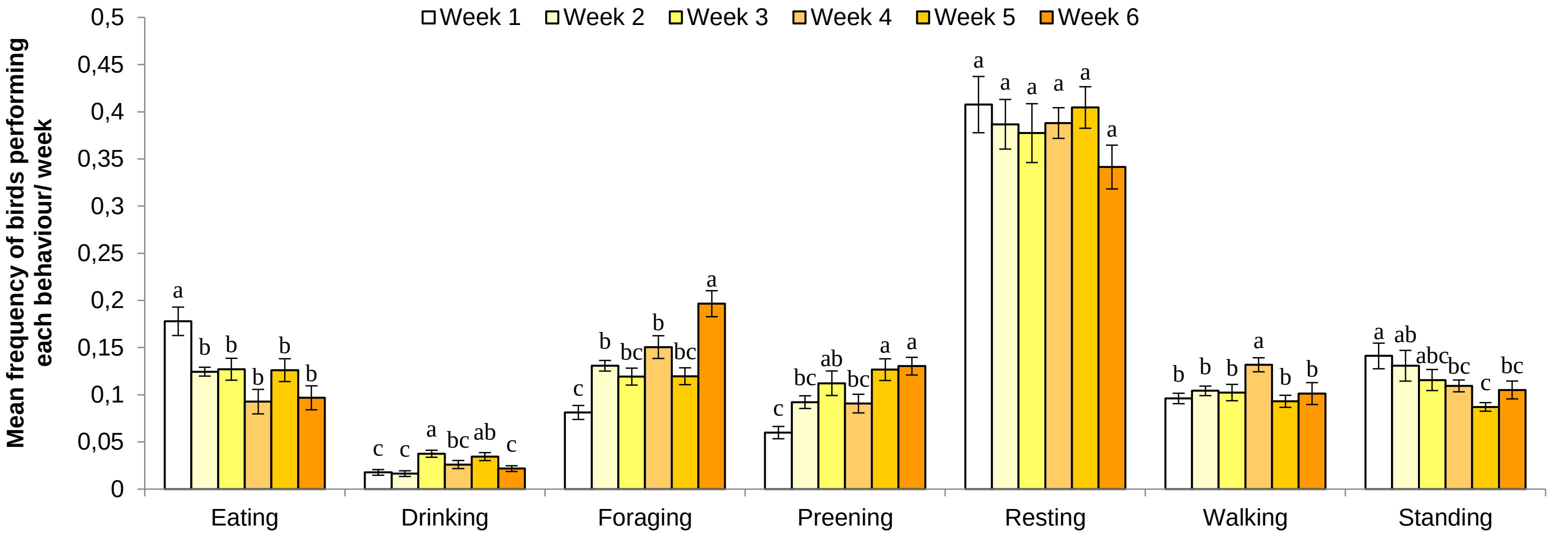


Figure 4.9 *Mean (± SE) frequency of birds performing each behaviour by week.* $^{(a\text{-}c)}$ *letters indicate significant differences across weeks for eating, drinking, foraging, preening, resting, walking and standing.*

4.4.6 Use of space

An effect of week of age was detected in the CENV pens, with birds using the floor less and the raised areas more with increasing age (Floor: $F_{5,10}$= 9.91, p=0.001; wooden blocks: $F_{5,10}$= 6.22, p=0.007; hide area: $F_{5,10}$=3.36, p=0.05; platform over hide area: $F_{5,10}$=50.26, p<0.007; and perches: $F_{5,10}$=59.99, p<0.0001; Fig 4.10). No effect of CSTR/NSTR treatment was observed, but there was an interaction between CSTR/NSTR and week of age for the use of the platform over the hide area ($F_{1,5}$=6.41, p=0.006; Fig 4.11) and a tendency for the same interaction in the use of perches ($F_{5,10}$=3.15, p=0.06, Fig 4.12).

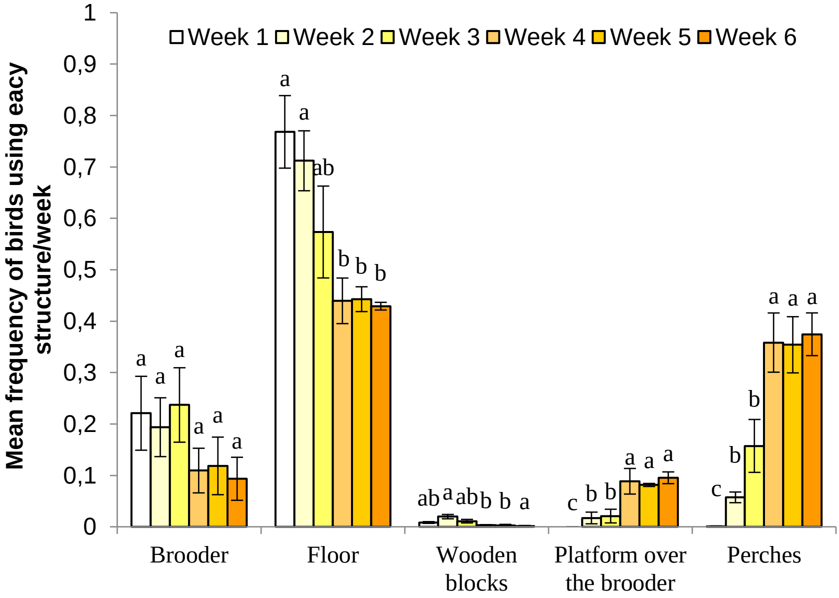


Figure 4.10 *Use of different areas in the complex environment treatment; Mean (+ SE) frequency of birds using the different areas of the pen by week in the CENV treatment;* $^{(a-d)}$ *letters indicate differences in the use of the structures (P<0.05) across weeks.*

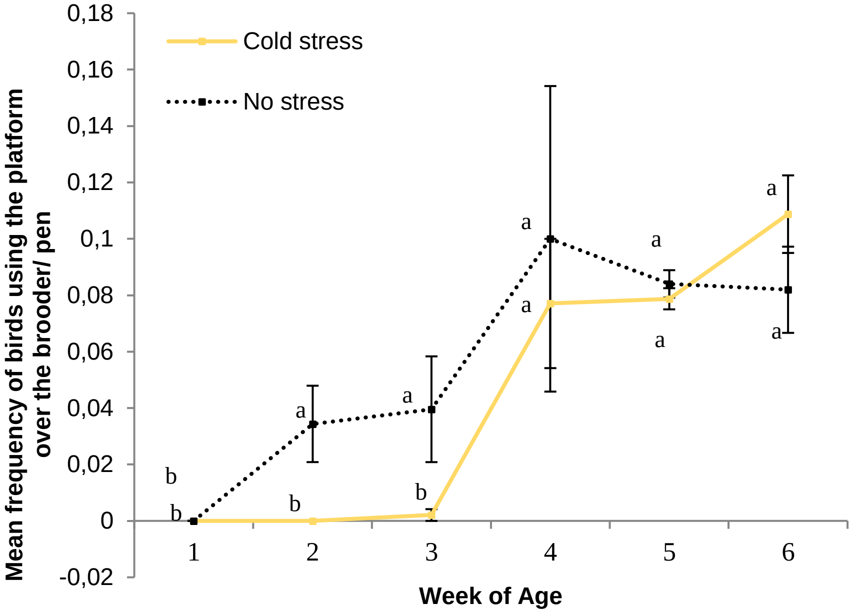


Figure 4.11 *Use of the platform over the hide area for CSTR and NSTR birds. Mean (+ SE) frequency of birds using the platform over the hide area for the Cold Stress (CSTR) and No Stress (NSTR) treatments across different weeks; $^{(a\text{-}b)}$ letters indicate differences within each treatment across weeks; no differences among treatments were observed in post hoc comparisons regarding each observed week.*

We also found an effect of week of age ($F_{5,15}$=18.56, p<0.001), perch level ($F_{3,6}$=21.80, p=0.0013) their interaction (week of age by perch level) on the mean frequency of use of perches ($F_{15,45}$=5.41, p<0.0001). Birds started by using the lower perches, but they used the higher perches more often as they got older. No effect of CSTR/NSTR or the interaction CSTR/NSTR by week of age was observed.

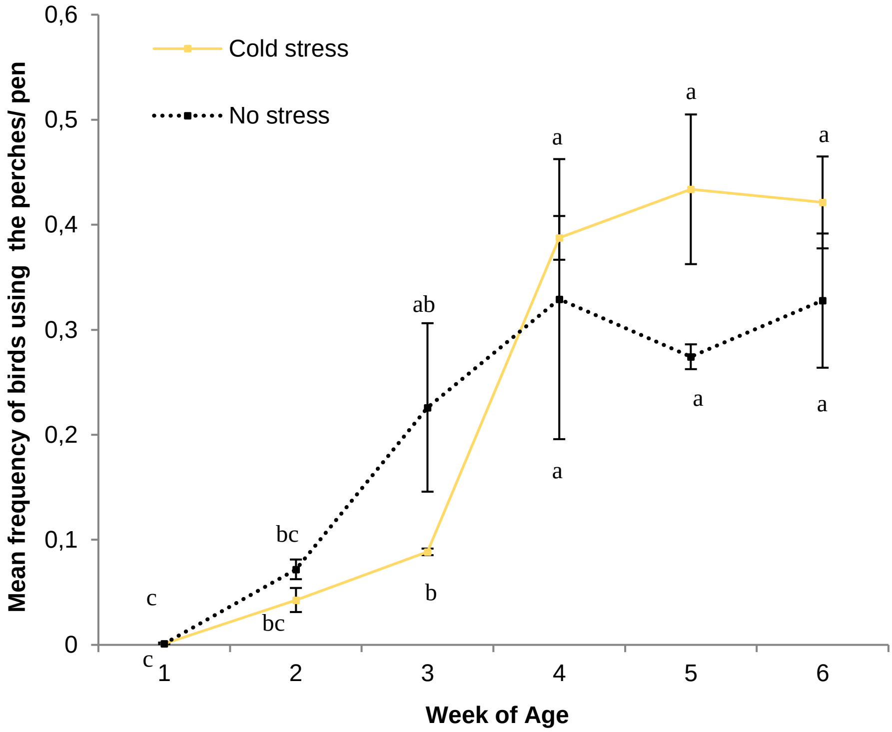


Figure 4.12 *Use of perches by CSTR and NSTR birds. Mean (+ SE) frequency of birds perching for the Cold Stress (CSTR) and No Stress (NSTR) treatments across different weeks; $^{(a\text{-}b)}$ letters indicate differences within each treatment across weeks; no differences among treatments were observed in post hoc comparisons regarding each observed week.*

4.5 Discussion

Our main results support the hypothesis that providing chicks with a more complex environment can help them improve some immunological and behavioural responses related to early stressors, as well as preparing them for future challenges. More precisely, we found that a CENV can ameliorate the effects of CSTR, allowing birds to express a higher pro-inflammatory response against PHA-P than their conspecific reared in a SENV (the first time PHA-P was administrated). Although there were no beneficial effects of the CENV initially, when analysing the H/L ratio during the three weeks directly after the CSTR there was a later buffering effect. Birds in the CENV had a lower H/L ratio following exposure to the ISCP compared to those reared in the SENV. No stress alleviating effects of the CENV environment after the ISCP were seen on birds' pro-inflammatory response to PHA-P or in their antibody response against SRBC. Additionally, but no less important, we found that the early CSTR experience, rather than improving chicks' capacity to cope with future challenges, actually seemed to make them more sensitive or vulnerable to them. This is based on their lower humoral response (antibody production against SRBC) and their slower start to use the elevated structures in the pen, such as the platform over the hide area and the perches. Although not all the consequences associated with the CENV are necessarily related to an immunological, behavioural and morphological improvement, the combined results from our investigation suggest that provision of a CENV early in life may contribute to improved welfare for poultry along different stages of their ontogeny.

4.5.1 Effect of CSTR/NSTR and CENV/SENV on physiological, morphological and immunological indicators

Considering that exposure to low temperatures is associated with increased energy needs for warmth, a reduction of the abdominal fat content was expected in pullets experiencing cold stress (Suk and Washbum, 1995; Yahav et al., 1996; Yunianto et al., 1997).

Consequently, CSTR birds might have been expected to have lower body weights, slower growth rates or poorer body condition than NSTR birds. However, we did not detect any difference between birds for any of these variables. The lack of differences could be due to the relatively short period of time (6h) during which the stressor was applied, or because birds may have recouped any loss of fat since food was provided *ad-libitum* once they returned to the pens. Additionally, we did not detect differences in the RFA of tarsus length and tarsus thickness. This would indicate that CSTR/NSTR birds probably experienced similar levels of stress, which is in accordance with the findings of Campo et al. (2007).

We observed that CENV birds showed a slightly slower growth curve in weeks four and five than birds reared in a SENV (Fig 4.6), but these differences disappeared by week six resulting in a similar weight for birds in both treatments. Birds reared in a CENV also had a slightly shorter tarsus length and lower body condition. These results might be a consequence of a higher level of exercise performed by birds in the CENV where there were perches and a platform. Increased exercise may be consistent with the fact that birds in the CENV showed more resting behaviour, although the furnishing in the CENV may also have made it easier for birds to rest undisturbed for longer.

Different scenarios were found for the different immunological indicators, the different stress sources and the time elapsed between stressors. The H/L ratios measured the day after birds were exposed to the acute cold stress were affected by the CSTR, as values were higher in CSTR birds than in their NSTR counterparts (Fig 4.7, T1). Previous research by Shinder et al. (2002) indicated that exposing birds to cold stress (15ºC during 3h) for the first time at three and four days of age resulted in a significant increase in plasma corticosterone concentration. The maintenance of an elevated plasma glucocorticoid concentration, induced by repeated exposure to stressful conditions, is known to reduce lymphocyte numbers and increase the number of neutrophils/heterophils (Dhabhar et al., 1995; Dhabhar et al., 1996). Our results indicate that acute cold stress during 6 h on only one day was sufficient to induce a physiological alteration affecting leukocyte distribution, similar to that observed with longer chronic stress exposures. In practice, such a stressor can occur when young chicks are transported from the hatchery to the rearing house.

No effect of housing environment (CENV/SENV) on H/L ratio was detected at T1. However, at that time, birds had been exposed to the environment for less than 24h. By the second time this variable was evaluated, birds were already 18 days of age (T2). We found that CSTR birds had significantly decreased their H/L ratio, reaching the values of their NSTR companions, which may indicate a time-dependent recovery phenomenon in this variable. Opposite to what we expected, no effect of the CENV was observed in T2, which may also indicate that recovery after CSTR was not influenced by the CENV. Directly after exposure to the ISCP (28-day old chicks-T3) we found a significant increase in H/L ratio, but only for birds reared in a SENV and independently of whether they had been exposed to CSTR early in life (Fig 4.7). It could be hypothesised that the CENV might have prepared the birds to cope with new challenges (such as those used in the

ISCP) and this is why birds reared in the CENV showed a reduced H/L ratio, indicative of lower levels of circulating stress response mediators. The acute CSTR applied in the first days of life seemed not to have had long lasting effects according to the results of the third sampling for this particular variable. Interestingly, the week after exposure to the ISCP (36 days old-T4) birds from all treatments had significantly increased their H/L ratio compared to the values observed before the ISCP (T2), but birds from the CENV treatment still showed significantly lower H/L ratios than those reared in the SENV. Increased environmental complexity, therefore, does have a positive effect on this immunological parameter.

Similar to our findings, increased H/L ratios have been observed in birds submitted to sources of stress such as fasting and frustration of feeding (Jones 1989), continuous lighting (Campo et al., 2007) or 1h of noise reproduced at 90dB (Campo et al., 2005), which confirms that these types of environmental stressors have negative effects on the immune system of domesticated avian species. Birds reared in the SENV had an immediate physiological stress response to the ISCP, inducing an elevation of the H/L ratio in T3, and they maintained it in T4. Given time, birds reared in the CENV seemed to cope with the ISCP since one week later they had only a small increase in the H/L ratio (lower than the increase observed in SENV birds).

An ameliorating effect of CENV was also detected in the response to the PHA-P injection at seven days of age (five days after the cold stress treatment). By this time CSTR birds reared in a CENV showed a swelling response similar to that show by

NSTR birds reared in either a SENV or in a CENV. In addition, this response was higher than that observed for their CSTR counterparts reared in a SENV (Fig 4.8). These results give additional support to our hypothesis that increasing environmental complexity is beneficial for the immune responses of stressed animals. Furthermore, it may provide information regarding how strong or long lasting the influence of CSTR may be. Based on the conditions used in this study, our results indicate that even five days after exposure to an acute cold stressor, the birds in the SENV were still affected by the consequences, displaying a diminished global pro-inflammatory potential.

However, by the second time PHA-P was measured (28 days old chicks, just after the ISCP) no ameliorative effect of CENV was observed. Even though by this time birds from the CSTR treatment and reared in a SENV were the ones showing the lower PHA-P response, all groups had a similar reduction in their inflammatory response. This could be explained in two different and not necessarily mutually exclusive ways. On one hand, it was the second time that birds were receiving the PHA-P injection, and second exposure to these types of antigens could induce lower responses. On the other hand, the effects of ISCP exposure on the birds' immune system may have been so great that it resulted in birds decreasing their response to a minimum, regardless of any environmental enrichment effects.

This repeated challenge issue is not applicable for the SRBC antibody response, since it was only applied once during the development of this experiment, after exposure to the ISCP. We observed that CSTR birds had a significantly lower antibody response than NSTR birds, which suggests that by the end of the experiment those birds exposed to CSTR early in life had their humoral immune responses altered. Similarly, Cichon et al. (2002) found that mice showed lower antibody production against SRBC after a chronic exposure to CSTR. Svensson et al. (1998) has also demonstrated that cold temperatures suppress antibody production in blue tits (*Parus caeruleus*). No ameliorating effect of the CENV was observed in our study regarding this variable.

Considering the results of our study, it is particularly informative to focus attention on the time points when the immune-related variables were sampled simultaneously. These experimental points were T3 (H/L ratio, PHA-P response and induction of SRBC response) and T4 (H/L ratio and SRBC response quantification). At T3, which was after the ISCP, birds showed diminished and homogeneous pro-inflammatory potential, together with an elevated H/L ratio if they were reared in the SENV. This is a potentially dangerous scenario. These birds had diminished pro-inflammatory potential and are also showing haematological indicators of a chronic stress response. Exposure to pathogens at this particular time, or near to it, would have been energy demanding and involve non-solved complications for the animals. Which may, unfortunately, have been what happened in this study for some birds. With regard to T4, and important to take into account in order to understand the situation at this point in time, is that birds had already been induced to provide a humoral antibody-mediated response in T3 (just after the ISCP). The diminished antibody response observed in the birds previously exposed to acute CSTR could therefore be indicating a cumulative effect of stressors along their ontogeny, because the non-stressed animals show no reduced antibody titres. The low H/L ratio at this time point supports (as previously mentioned) a better stress coping by CENV reared birds. This last point does not invalidate the fact that humoral responses were globally affected in CSTR and ISCP exposed groups.

Nazar and Marín (2011) reported that non-enriched/non-stressed quail had the lowest swelling response after PHA-P injection and the highest H/L ratio in comparison to the other treatments (enriched/stressed, non-enriched/stressed, non-enriched/non-stressed). Although, our results are quite similar regarding the pro-inflammatory response (PHA-P) we were not able to detect any ameliorative effect of CENV on the humoral component of the immune function (anti-inflammatory responses). One possible explanation for the differences across studies may be that in ours, half of the birds were submitted to two sources of stress (CSTR and ISCP), while in the study of Nazar and Marín (2011) all birds were submitted to a single chronic stressor (15 min of daily restraint). An important question to address in future research, therefore, is whether the difference in the humoral response of CSTR birds was produced just after CSTR or, later on, after the additional exposure to the ISCP due to an accumulative effect of stressors.

4.5.2 Effects of CSTR/NSTR and CENV/SENV on behaviour and use of space

No effects of CSTR were observed for the different behaviours analysed. This may reflect a lack of power in the study for those behaviours analysed at pen level. However, chicks reared in a CENV rested more and were observed to stand and walk less. Some differences in behavioural variables between the SENV and the CENV were expected due to the different nature of the resources provided. For example, previous research suggests that an increase in environmental complexity in the form of perches or cover decreases the number of disturbances from conspecifics and increases time spent resting (Newberry and Shackleton, 1997; Cornetto and Estevez, 2001ab; Cornetto et al., 2002). Resting is important, especially for young animals (Malleau et al., 2007), as it influences growth, energy conservation, tissue restoration and coping abilities (Bolkhuis, 1984; Zidar et al., 2018). If the provision of a CENV allows birds to rest more, it is probable that it also favours a better recovery after stressful events. This hypothesis is consistent with the part of our results regarding the ameliorative effect that the CENV had on some of the immunological variables, such as the first PHA-P injection and the H/L ratio after exposure to the ISCP. From a behavioural point of view, the provision of a CENV may have helped birds to cope better with new challenges also of a different nature. For example, provision of a CENV designed according to birds' behavioural needs (e.g. favouring their escape responses) would provide chicks with a certain possibility to control their environment, which might be important to lower their stress response (Koolhaas et al., 2011).

Interestingly, in a parallel study performed with the same chicks we found that birds from the CENV remained more optimistic after being submitted to the ISCP than those reared in a SENV (Zidar et al., 2018). This may indicate that birds from the CENV had a more positive affective state than those reared in a SENV, which could have helped counteract some of the stress-related deleterious effects. Previous studies in rats have already suggested that pessimism may make individuals more susceptible to stress (Rygula et al., 2013) which seems consistent with the combined results found in our studies.

The Red Jungle fowl uses high places for rest and escape from ground predators and this preference for higher perches can still be observed in domestic lines (Appelby and Duncan, 1989; Keeling, 1997; Newberry et al., 2001; Odén et al., 2002; Wichman et al., 2007; Struelens et al., 2008; Schrader and Müller, 2009). Our results are in accordance with this. As the birds' physical abilities and strength increased with age, they tended to spend more time on the higher perch and less time on the lower structures (Fig 4.10, Fig 4.11 and Fig 4.12). Resting as far as possible from the ground may give birds a greater sense of security (Brake et al., 1994; Keeling, 1997; Newberry et al., 2001) and may also have helped them recover after exposure to stressors.

An effect of the interaction between CSTR/NSTR treatment and week of age was found when analysing the use of the platform over the hide area and the use of perches. Our results indicate that CSTR birds had a slower development in their pattern of use of these elevated structures compared to NSTR birds (Fig 4.11 and 4.12). However, in the case of the use of perches, probably due to the low number of replicates, the post hoc results were inconclusive. According to the adaptive plasticity theory, the input individuals receive during their development may have long lasting effects on the modulation of their phenotypes (Nettle and Bateson, 2015). Following this argument, we speculate that CSTR birds could have developed a phenotype that expresses more anxiety-like behaviour due to the input they received at an early age and that this led to them being more fearful in the exploration of their environment. Supporting this, and reported in the previously mentioned parallel study by Zidar et al. (2018), CSTR birds were faster to first step in a detour social reinstatement test than NSTR birds after being exposed to the ISCP, which may be indicative of higher anxiety-like behaviour. It has also been shown that CSTR birds have longer tonic immobility duration, indicating higher fear levels (Campo et al., 2008). At least in the conditions of this study, CSTR early in life seems to have resulted in a phenotype that was less well adapted to exploiting the potential of the CENV.

4.6 Conclusions

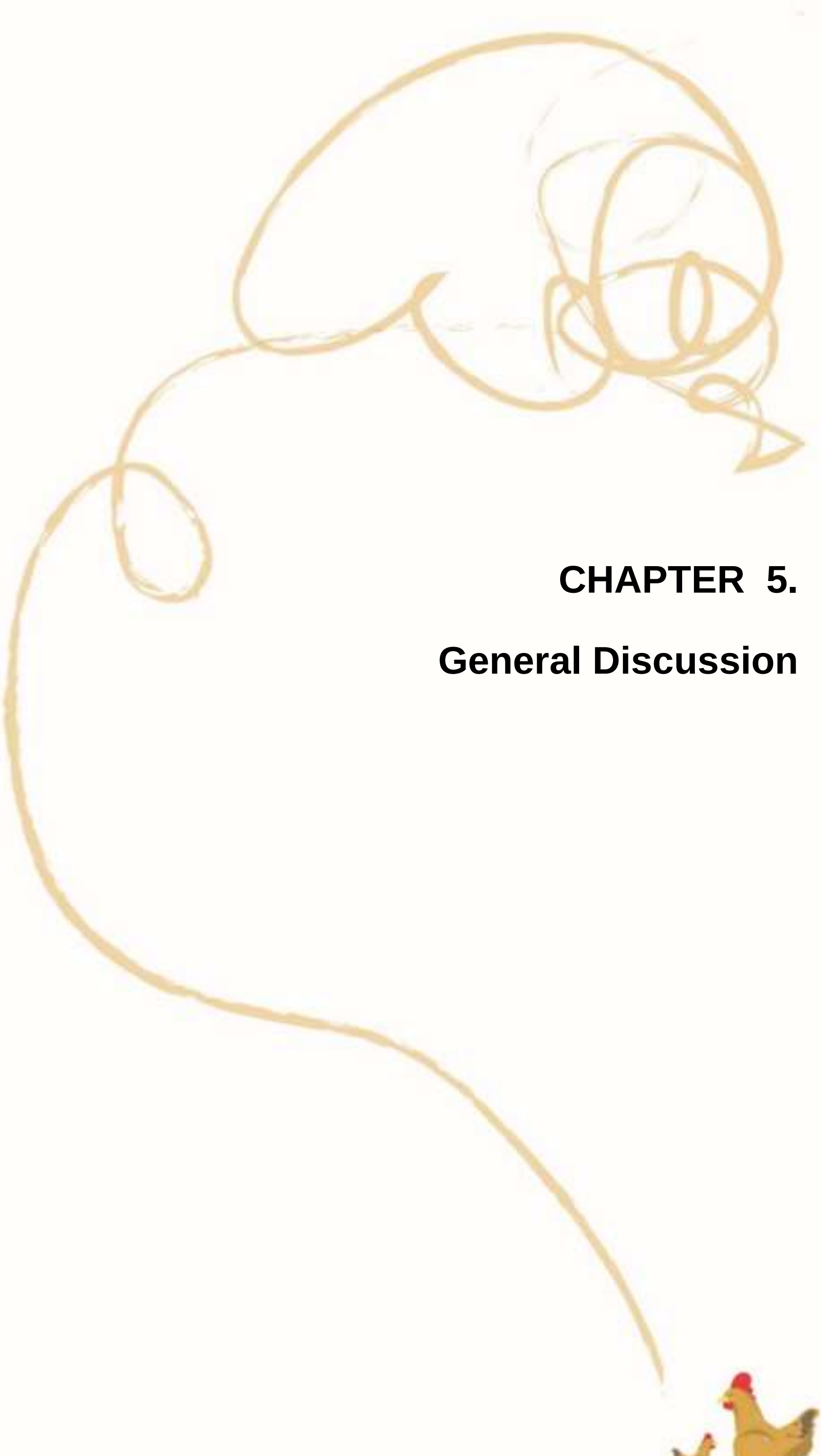
This study demonstrates that environmental complexity during rearing may not only help to ameliorate the consequences of early stressful events, but may also help birds to cope better with future challenges. Even though not all the immunological or behavioural stress impaired responses were mitigated by the provision of a CENV, some of them were improved when compared with the responses observed in birds reared in a SENV. We could interpret our results in the context of the adaptive plasticity theory. The different inputs received by the birds in early life (cold stress or environmental complexity) would have differentially affected them, configuring potentially different phenotypes. Birds with a CENV as an input may have a better physiological response against stress than birds reared in the SENV. This would suggest that the wider possibilities to control their environment, and their physiological responses, for example by resting in a quiet area when needed or having higher activity possibilities, would mean that the stress affected them less or made them better able to develop stress coping strategies. Additionally, we determined that acute cold stress during early life may have short- and long-lasting effects depending on: i) the variable analysed, ii) whether there is a series of different inputs during ontogeny (e.g. an additional challenge such as an ISCP) and iii) the characteristics of the rearing environment. Given that even under good husbandry conditions, the rearing of laying hen pullets is never going to be stress-free, this research has contributed to increasing our understanding about why the provision of a complex environment is crucial for improving their welfare.

Acknowledgements

We wish to thank Helena Jones, Rebecca Katajamaa and Sonja Broberg for their excellent help during the development of the experiment. This study was carried out within the Centre of Excellence for Animal Welfare Science, a Swedish collaborative research platform.

CHAPTER 5.

General Discussion



5.1 The social environment

The main objective of this Thesis was to evaluate the impact of different social and environmental challenges on the welfare of laying hens at different stages of their ontogeny. In Chapters 2 and 3, the effects that altered phenotypic appearance (PA) had on the social interactions of laying hens during two distinct periods was assessed: In young laying hens (pre-laying period; Chapter 2); and during adulthoods (after laying was well established; Chapter 3). The results obtained indicate that the effects of PA alteration differed considerably according to the stages of laying hens' ontogeny. While the effect of PA on social interactions of pullets was minimum during rearing, when laying hens reached adulthood the effect had an important impact on aggression rates.

In the study corresponding to Chapter 2, we expected that birds with altered phenotypes would have a greater probability to become targets of aggression (Estevez et al., 2003; Dennis et al., 2008) and lower probability of engage in affiliative interactions (Hamilton, 1964a, b; Lizé et al., 2006). Contrary to our predictions, we did not find clear effects of altered PA on aggressive or affiliative interactions in pullets. The lack of PA effects observed could be explained considering imprinting or an early filial learning process (Lorenz, 1935; Bolhuis and Bateson, 1990; Bolhuis and Honey, 1998). Imprinting mechanisms are deeply integrated in many species as they determine early social learning and will affect to their chance to survive (Versace and Vallortigara, 2015). Close to the imprinting window it is when, according to phenotype matching mechanisms, the recognition templates that will be later used to distinguish between familiar and unfamiliar individuals are developed (Bateson, 1979; Bateson, 1990; Gerlach and Lysiak 2006). Therefore, it could be possible that the birds in our experiment would have ´assumed´ the phenotypic diversity of the group (or the lack of it). In this sense, birds could have created their template for the recognition of familiar individuals in a more or less flexible way according to the conditions of the early social environment to which they were exposed, specifically: the phenotypic diversity they grew with. However, these results contrast with previous investigations carried out in broilers and white leghorn layers of a similar age and where clear effects of the alteration of the PA were detected (Estevez et al., 2003; Dennis et al., 2008). The differences observed could be related to the fact that the black mark used in our study over a brown plumage was less conspicuous than the black mark used by Estevez et al. (2003) and Dennis et al. (2008) over white plumage (Chapter 2). However, when alteration of the PA took place in adult laying hens (Chapter 3) the results obtained were similar to those mentioned. In adulthood, the same alteration of PA (black mark at the back of the head) performed in our originally homogeneous groups of birds (100U, U: Unmarked, by altering the 30% of the birds in the group) created a drastic increased in aggression that was directed towards the newly marked (M) birds (Fig 3.4). Moreover, laying hens originally from 100M groups, 30% unmarked (U), were also attacked at similar levels by their unaltered marked (M) pen-mates. These results suggest that conspicuousness of the mark may be a factor that influences how easy a phenotype would be accepted during early rearing. However, once the temporal window for imprinting has passed phenotypic changes cause a deep impact, dramatically increasing targeted aggression over a specific bird phenotype and, thus, compromising the welfare of such birds. The fact that laying hens seem to be more reactive to changes in their social context during adulthood indicates that this might be a period where PA and aggression should be monitored for better management decisions. At the productive level, increased aggressive interactions can be triggered by different factors such as diseases, injuries, accidents or even delay in the offset of laying that make the birds change in PA during adulthood causing important welfare problems, even involving economic losses due to the risks associated.

As we observed during rearing, pullets showed a greater facility to accept phenotypic diversity in their social environment. This is probably because birds can increase social plasticity in early ontogenic stages. Contrarily in adulthood, this capacity may disappear and birds would become more reactive to changes in their close social context. During adulthood, template recognition for phenotype matching processes must already be well established to allow the accurate recognition of unfamiliar individuals who may represent risks to the local population. The reason for this increased reactivity observed in adulthood may be the result of their particular evolutionary history. Such a strong and well-structured reaction towards individuals with a different PA could have protected birds from potential invaders that could out-compete locals for the available resources (Barnard, 1991; Hurst and Barnard, 1992) or even from individuals that could be vectors in disease transmission (Goodall, 1986; Lewis, 1998; Hughes and Cremer, 2007). The costs in fitness that can result from not responding effectively to unfamiliar phenotypes can be high and even result in death.

It may be questioned why the birds in the studies presented in this Thesis responded so strongly to artificially introduced signals. This could be due to the fact that the involved costs of not responding to the signals effectively when can, in fact, be real are greater than the costs of responding to them when the information they contain is not honest (Mokkonen and Lindstedt, 2016). Taken all this together it is interesting to note how complex the social responses of laying hens can be, allowing them to respond differently depending on the chain of events taking place during development. Phenotypic plasticity is defined as the capacity of individuals to adaptively alter their phenotypes as adults and is specifically referred to intra-individual changes (Piersma and Drent, 2003). Thus, we may argue that during adulthood laying hens presented phenotypic flexibility as they were able to adapt their behaviour to the changing conditions of the social environment. This term “phenotypic flexibility” is framed in terms of trade-offs as a way to explain the extent to which phenotypes may vary in response to the environment (Piersma and Drent, 2003). Consequently, we may argue that when the social environment changes during adulthood, laying hens may invest more in a “defensive phenotype” to actively protect themselves from potential intruders, because the costs intruders can involve are larger than the benefits derived from not expressing such behaviour. We also predicted that the effect of modifying the PA would be diluted as the percentage of altered individuals increased (Dennis et al., 2008), as well as in larger groups, since both imply an increment in the number of birds potentially becoming a target for aggressive interactions (Marin et al., 2014). We did find a reduction of aggressive and affiliative interactions as the size of the group increased in pullets (which will be commented later on). This effect, however, was independent of PA as we did not find a dilution effect of aggressive or affiliative interactions as the proportion of altered birds increased. Nevertheless, in our experiment at early ages, we detected a combined effect of GS by type of interaction (MM, MU, UM, UU). In groups of 20, a higher than expected aggression rate was detected from unmarked (U) directed towards marked (M) individuals. This finding might be due to the combination of unmarked birds to start an interaction and the number of marked birds to receive it.

We expected that changes in phenotype appearance in adulthood (Chapter 3) would have more severe effects, especially when the proportion of altered individuals was low (Dennis et al., 2008). According to our prediction, the highest aggression rates were detected when the lower proportion of birds 30% was altered in originally homogeneous groups (weeks 35-36). Aggression in these cases was clearly directed towards the altered birds, whether it was in homogenously marked groups (M) in which altered birds were unmarked, or unmarked groups (U) in which altered birds were marked. As the proportion of altered individuals increased to 50% (weeks 39-40), aggression started to decrease, reaching levels close to the original (before alteration) when 70% of the group were altered (weeks 45-46). Although aggressive interactions decreased notably by the time most of the group was altered, the interactions remained clearly directed towards altered individuals. These results, overall indicate that despite the social turmoil created by the initial alteration (30% altered), birds were able to adapt over time to the changing social conditions by incorporating the emerging phenotype within their acceptable templates, even during adulthood. It seems then, that laying hens are reassessing their social environment constantly to make strategic decisions based not only on what they perceive, but also on the information they gain by interacting with their mates. We could speculate, that laying hens were able to detect that the emerging phenotype was no longer a risk, or simply, if the number of altered phenotypes increase the effects would get diluted with more birds being targeted by fewer birds from the original phenotype. Specifically, in each consecutive PA change, more individuals share the cost of displaying the altered phenotype, at the same time that fewer individuals remain unaltered as possible initiators of interactions (Marin et al., 2014). This would in turn be translated in a reduction of aggression as the proportion of altered birds in a group increase (from 30-50-70%).

In adulthood (Chapter 3), it was expected that the impact of phenotypic manipulation would be less remarkable in small groups of 10, in which individual recognition it is assumed (Bradshaw, 1992), as compared to large groups (40) where such recognition was predicted to be more difficult (McBride and Foenander, 1962; D'Eath and Keeling, 2003). Contrarily, the effect of PA alteration was not mitigated by the potential capacity of individual recognition assumed in small groups after altering the PA of the 30 and the 50% of the birds. Only in the last PA change altering the 70% of the group, an effect of GS was detected. In this case, larger groups of 40 showed somewhat higher aggression levels compared to small groups with 10 birds, while groups of 20 showed non-significant intermediate values. This suggests that larger groups might have taken longer to return to the levels of aggression similar to that observed before applying phenotypic changes, results that might be interpreted as a sign of higher difficulties of re-stablishing social order and adapt to phenotypic alterations. However, this hypothesis should be further explored in the future.

5.2 Effects of Group Size

Regarding the previously mentioned effect of group size (GS) on social interactions, results indicate that young laying hens (Chapter 2, Fig 2.1 and 2.2) reared within small groups of 10 individuals showed a higher frequency of social interactions both negative (in the form of threats) and positive (in the form of exploratory feather or beak pecking) than in intermediate (20) or large groups (40). Although, this Thesis was not focussed on exploring the effects of GS alone, the results obtained are consistent with previous research. Domestic fowl as well as its wild ancestor, the red jungle fowl, when kept in small groups tend to establish a hierarchical social system (Collias et al., 1966; Queiroz and Cromberg, 2006). It is expected that the number of social interactions required to establish such hierarchical system would tend to increase as the number of birds in the group raise. However, an alternative hypothesis suggests that aggression would increase until reaching an inflection point, from which it starts decreasing because the costs of establishing a hierarchical system would have outweighed the benefits derived from it (Estevez et al., 1997; Pagel and Dawkins, 1997). Research have shown that such inflection point can lay close to groups of 30 individuals which are the most problematic, as they are considered socially unstable, whose members produce eggs of smaller size and present a lower body weight (Keeling et al., 2003). If this was to be true, groups of 40 individuals would be too large for the hierarchical system to be stablished. Consequently, aggression would be low due to the adoption of tolerant social system (Estevez et al., 1997, Estevez et al., 2003). As GS of 20 showed intermediate values of aggression the inflection point could not be detected in this case, but it could coherently be argued that it should be near 20.Consequently, the results obtained do not deny the formation of a social hierarchy in small groups (Schelderup-Ebbe, 1922; Wood-Gush, 1971; Rushen, 1982) or the adaptation of a tolerant system in larger ones (Estevez et al., 1997; Estevez et al., 2003).

An alternative explanation for the results obtained in Chapter 2 regarding the effect of GS could be proposed. This explanation would be based on the idea that pullets in different GS, live in enclosures with different spatial characteristics, which in turn may have determined differences in birds´ movement patterns and their interaction probability. Although maintaining a constant density in all studied GS, in small groups the space available was less effective as compared with large groups housed at identical density. Total space is larger, the larger the group and, therefore, birds are able to move more without bumping into each other. Such differences in spatial movement patterns at different GS and constant density that would explain differences in behaviour have been demonstrated for broilers and laying hens (Leone and Estevez, 2008; Liste et al. 2015). This hypothesis, gains support in our study because it explains both results on aggressive behaviours (threats) and those related to affiliative interactions (beak pecking and explorative feather pecking). If the previous contention is true, we wondered why we did not register the same pattern in adult layers at T0 if the space was the same and the birds were larger. Reasons that could explain the differences in adult birds at 27-28 weeks of age could relied on; 1) the characteristics of the studied ontogenic stage and/or 2) the differences in the experimental procedures. During adulthood, birds probably reduced their interaction frequency because they already knew their social environment. It is assumed that by 27-28 weeks of age the social system (either hierarchical or tolerant) would already be functional and established. Consequently, the level of interactions necessary to maintain its functionality is expected to be low and stable (Queiroz and Cromberg, 2006). It is during early rearing that birds discover their social environment developing a particular type of social organization. When the social system is being worked out (8-12 weeks of age; Rushen, 1982), a greater number of social interactions are expected. This could be the underlying reason for the differences in results of GSs across age. This, of course, would be a hypothesis that will require further investigation. On the other hand, it should be highlighted that aggression has been computed differently in the two studies performed. In the first study Chapter 2 aggression was separated in two different categories: threats and sum of aggressive pecks, leaps and chases. GS only had a significant effect on threats, the most frequently observed aggressive interaction. During adulthood (Chapter 3), aggression was condensed in a single variable for analysis (grouping threats, aggressive pecks, leaps and chases), as their frequency was lower. Nevertheless, the grouping as was done consistently across GS would have detected differences if there was any. It is also important to mention that the dynamics of affiliative interactions were not studied during adulthood. This could have provided valuable information to clarify if the effects of GS on affiliative interactions persist until adulthood. However, when testing the effects of altering the PA in adulthood the frequency of aggressive interactions increased tremendously. It would have not been feasible for a single observer to register the different ranges of affiliative and aggressive behaviours at the same time and videorecording did not allowed us to keep the ID of the birds giving and receiving the interaction, which was essential for the study. Consequently, we focused on aggressive interactions during adulthood.

To conclude the first part of the Thesis, our results suggest that laying hens respond differently to different social context depending on their ontogenic stage. When young, their social interactions were greatly influenced by the size of their group, or determined by the size of their enclosures, while PA effects were minimum and probably had an imprinting base. Contrarily, as adults, laying hens were much more sensitive to PA changes of their conspecifics, which in turn could lead to major welfare issues. These results contribute to a better understanding of the complex communicational system that layers use for building their social system and adapting the way they socially interact as an answer to the changing conditions of their environment. These results concur with the idea that the social organization for laying hens is, more plastic and dynamic than previously suggested. The results obtained have a practical application for the egg industry, as they highlight the importance of the early detection of those individuals that, through the production cycle, can present changes in their phenotypic appearance (due to injuries, wounds or differences in food intake), and therefore are at higher risk of becoming targets of systematic aggression by their conspecifics. Building tools that allow us to avoid the appearance of such problems or improve methods for the early detection and isolation of these individuals will help to avoid escalated aggression in our flocks, as well as, anticipate further problems. This in turn, would contribute to design better welfare protocols for laying hens' production systems.

5.3 The relevance of the early environment

Regarding the second part of this Thesis, in Chapter 4, we predicted that the early provision of a complex environment (CENV) would attenuate the effects of early stressors as well as buffer against the future unpredictable challenges. The results obtained suggest that providing pullets with a CENV from early ages can benefit their immunological and behavioural responses to early stressors, as well as prepare them to better cope with future challenges. More precisely, our results indicate that provision of CENV contribute to ameliorate the effects of cold stress treatment (CSTR), allowing birds to express a greater pro-inflammatory potential than their conspecifics reared in a simple environment (SENV). Although, we did not observe beneficial effects of CENV at the beginning of the experiment (weeks 1 and 3) for the H/L ratio (no alleviation of the effects from early CSTR treatment in this variable), when introducing a second stress period (by exposing birds to an intermittent stressful challenges protocol (ISCP)) birds from CENV presented a lowered H/L ratio compared to their mates reared in a SENV the same week of ISCP exposition (week 4) and the last week of the experiment (week 6). This in turn, may be indicative of a better response to ISCP from birds reared in the CENV. However, we did not find any ameliorating effect of the CENV after the ISCP when measuring for second time the pro-inflammatory response, neither when assessing birds' humoral response during the same period. It is important to clarify, that the second time PHA-P was administered the response in all groups was homogeneously low. This could have been due to the fact that: 1) the second exposure to the same type of antigen is usually lower (Davison, 2014) or 2) because the effect of exposing pullets to the ISCP may have been so strong that it reduced the response of the animals to a minimum whether or not they were reared on a CENV. The exposition to ISCP may, at this time, imposed such a high demand of resources that pullets would have responded minimizing their response in order to be able to maintain body system functionality.

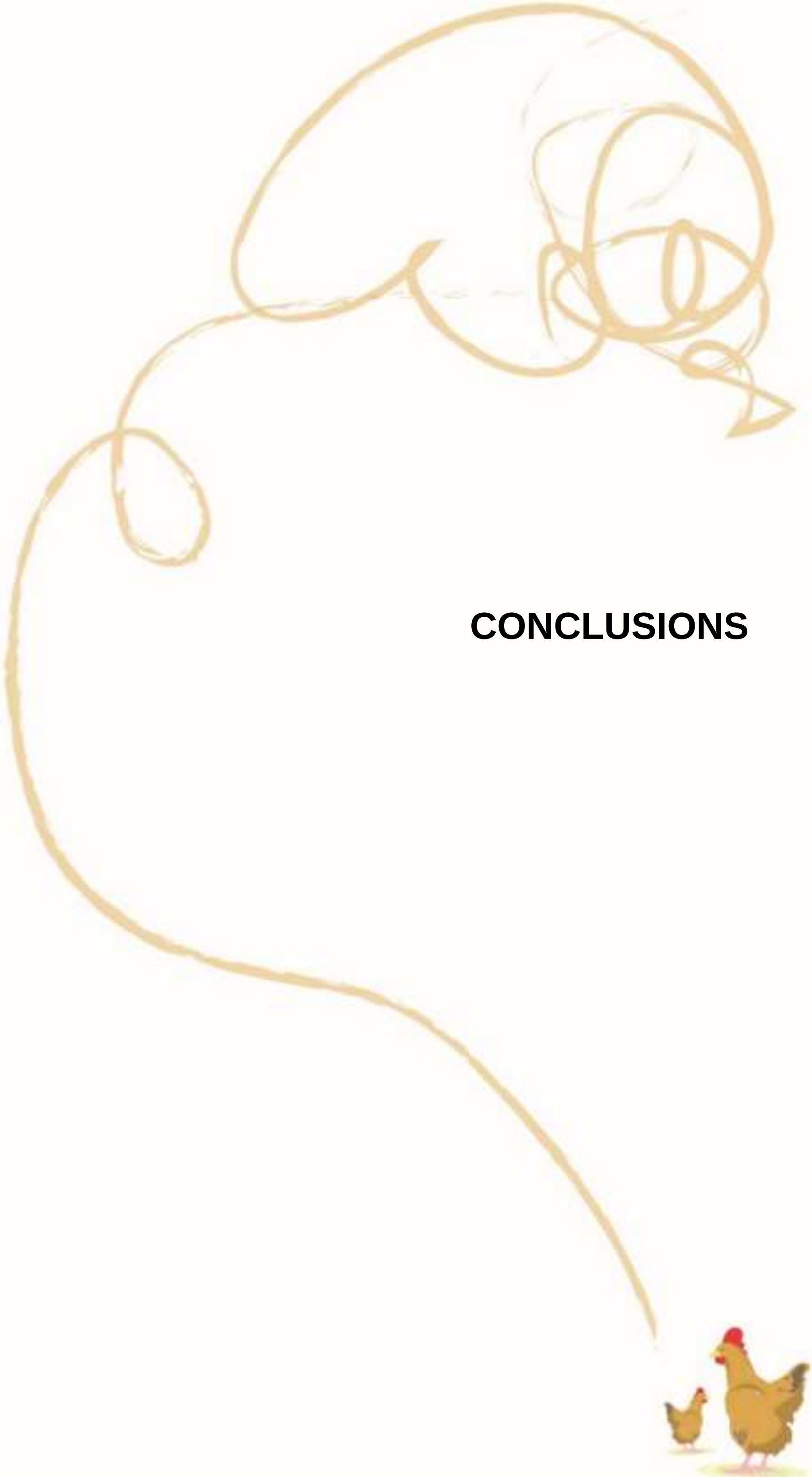
The main improvement detected regarding the CENV in behavioural variables was that it offered greater possibilities to rest without interruption, as seen in previous research (Newberry and Shackleton, 1997; Cornetto and Estevez, 2001a, b; Cornetto et al., 2002; Leone et al., 2007). This, in turn, may be potentially associated with better stress recovery from birds living in CENV, as indicated by the results obtained regarding the immunological variables mentioned above. Resilience is defined as the capacity of animals to cope with short-term perturbations in their environment and return rapidly to their pre-challenge status (Colditz and Hine, 2016), thus the better stress recovery observed in CENV may have been associated with higher resilience. Likewise, the CENV provided in this experiment, may have offered a space better adapted to the behavioural needs of the birds, since it favoured the expression of a wider behavioural repertoire (it allows jumping up and down of the perches and platforms or hiding in dark places such as the dark brooder). These introduced features may have promoted the expression of the basic anti-predatory responses from layers (such as climbing to high places or hiding). Increasing CENV may have allowed pullets to develop a sense of control over their environment (Keeling, 1997), which may have contributed to mitigate the intensity of their stress responses (Koolhass et al., 2011). In a study carried out in parallel with the same pullets, we detected that rearing in this CENV allowed birds to remain more optimistic after being subjected to ISCP and compared to pullets reared in SENV (Zidar et al., 2018). These results suggest that the provision of a CENV not only provided physical health benefits for the pullets, but it could even improve their affective state. This is of crucial importance, as it seems that the provision of a CENV may involve a multilevel improvement for laying hens' welfare. The provision of a CENV appear to have influence at least on three of the ´Five Freedoms´ proposed for improving animal welfare: it provides an appropriate environment including shelter and a comfortable resting area (freedom from discomfort); it provides sufficient space and proper designed facilities (freedom to express normal behaviour); and, it ensures conditions that may help to avoid mental suffering (freedom from fear and distress).

Furthermore, we observed that exposure to an initial period of CSTR, instead of improving individuals' ability to face future challenges, it renders them more vulnerable or sensitive. This idea is based on the evidence that individuals that had undergone CSTR early in life, presented a lowered humoral response (antibody production against SRBC) and a slower use of elevated structures (perches and platforms) in CENV. These results are in accordance with the previously mentioned study by Zidar et al. (2018), which also found that CSTR birds presented a more anxiety-like behaviour in a detour test after being exposed to ISCP than their NSTR mates. However, form the present investigation, it remains unclear whether the lower production of antibodies in response to the SRBC injection, was due to the effect of CSTR experienced early in life, or if it was a consequence of the cumulative effects of subjecting CSTR pullets to a second ICSP stress period. Alternatively, low antibody production has been associated sometimes with fitness benefits because the production, use and activation of the immune system they involve many times is a highly demanding task (Lee, 2006).

Chapter 4 concludes that although not all the consequences associated with living in a CENV respond to a behavioural, immunological or morphological improvement of the stress response, some of them do. Consequently, the combined results extracted from this chapter suggest that early provision of a CENV in the early rearing environment of laying hens can help to improve their long-term welfare throughout production. If we interpret these results in the context of adaptive plasticity theory, the different inputs that birds receive early in life (cold stress and environmental complexity) may have affected laying hens in different ways, configuring different final phenotypes. Birds reared in a CENV as an input may have developed a better physiological response to stress than birds reared in a SENV. However, birds submitted to CSTR early in life would be more sensitive in future in challenging situations such as the exposition to ISCP.

As a global conclusion of this work, the abilities that pullets acquire during early ontogenic stages will help them in subsequent stages, improving their probability for better coping when facing new challenges, thus ensuring/elevating their welfare status. This is why exposing young laying hens from an early age to greater social diversity and/or to increased environmental complexity, may offer them more opportunities to respond appropriately to a changing social environment or to cope with stress in future situations. This is because laying hens not only learn and imprint on their social context but they also do to the environment in which they live. After performing this work, we propose that as responsible members of society it is necessary to broaden our vision when designing production systems or making management recommendations. In this sense, if we consider designing environments well-suited to ensure high welfare standards for laying hens, consideration must include the quality of life that they experience throughout the production cycle as one single process, not separated by phases as it is normally viewed. Further research should focus on alternatives that make possible to obtain highly plastic and adaptable birds, which will be better able to deal with changing environmental conditions. Therefore, providing them with appropriate social groups and with environments that can favour their physical, mental and emotional health is crucial. Additionally, the stimuli that domestic fowl experience throughout ontogeny, either physical or social, will shape the possibilities and potential response that birds will show, affecting to specific traits of each individual in a particular way. In turn, these possibilities and potentialities will determine the quality of life of each individual. Therefore, the inputs that laying hens experience along their life will have a direct effect on their welfare.

CONCLUSIONS



CONCLUSIONS

This work provides important insights in the study of the effects that different social and environmental challenges have on laying hens' welfare during different phases of their ontogeny. The main conclusions extracted are:

1. The effects of phenotypic appearance (PA) on aggressive and affiliative interactions in pullets were minor as the PA treatment did not affect any of the mentioned behaviours. These results were probably associated with the imprinting process that favoured a good integration of the altered phenotype in heterogeneous groups at this age.

2. Contrary of what we observed for pullets, the emergence of new phenotypes in originally homogeneous groups of adult laying hens produced an escalation of aggression clearly directed towards birds presenting the new phenotypes. This aggression may be used in order to avoid the potential threats implied in these new phenotypic configurations.

3. Directionality of aggression during adulthood was equally observed when birds were marked in a homogeneous unmarked population (increasing conspicuousness), and when birds were unmarked in a homogeneous marked population. Phenotype matching mechanisms should be considered as a possible explanation to the reaction observed to new phenotypes.

4. Although, for young laying hens the proportion of altered birds did not affect any of the behavioural variables explored, during adulthood, a dilutive effect of aggression was found in the groups as the percentage of altered birds increased (from 0-30-50 and 70% of the group altered). This last result was associated to the fact that, in each alteration, the number of possible initiators of the aggression (unaltered birds) increased while the number of possible targets of aggression (altered birds) decreased.

5. During rearing, pullets from small GS of 10 individuals socially interacted more than those from groups of 40 (performing more affiliative and aggressive interactions). The frequency of interactions may be modulated by the characteristics of the space offered and the probability birds have of running into another individual.

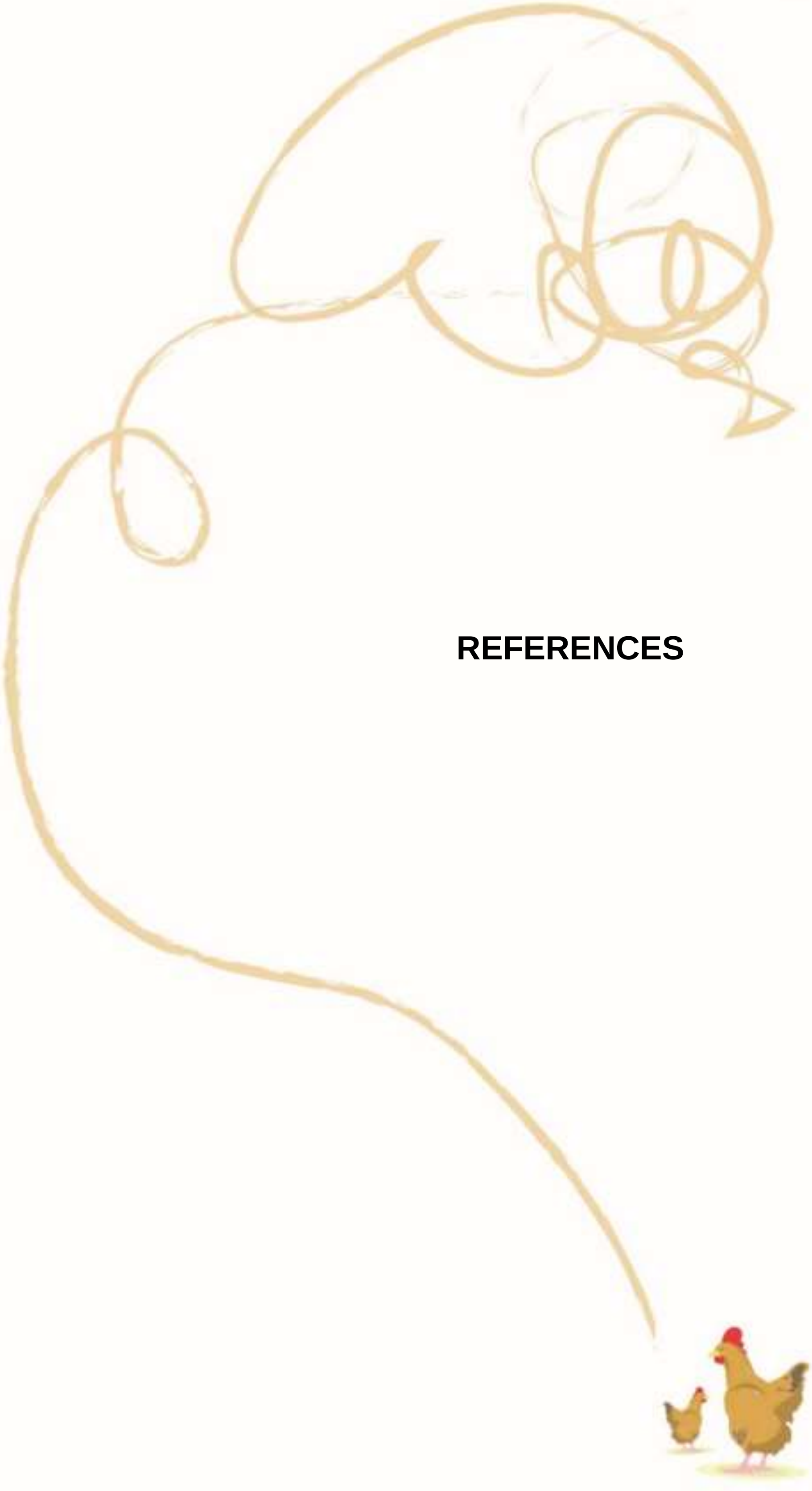
6. Only marginal effects of GS were detected during adulthood on the analysed variables, suggesting that the impact of PA over aggression rates was far more important to grant social stability at this stage.

7. According to the adaptive plasticity theory the different inputs received by laying hens in early life (cold stress (CSTR) or environmental complexity (CENV)) would have differentially affected them configuring potentially different capacities to react against the challenges.

8. Birds with a CENV as an input may have a better physiological response against stress than birds reared in

the SENV. The provision of CENV during rearing may not only help to ameliorate the consequences of early stressful events, but may also help birds to better cope with future challenges.

9. Birds that experienced CSTR during early ages may be more vulnerable to future challenges as their humoral responses were lower and they start using elevated structures in CENV slower than non-stressed (NSTR) pullets.
10. Overall, the results obtained during the development of this Thesis suggest that early social and environmental conditions offered during rearing may be crucial to ensure a good welfare standard for production conditions in poultry.



REFERENCES

REFERENCES

A

Abeyesinghe, S.M., McLeman, M.A., Owen, R.C., McMahon, C.E. & Wathes, C.M. (2009). Investigating social discrimination of group members by laying hens. Behavioural Processes, 81(1), 1–13. https://doi.org/10.1016/j.beproc.2008.11.017

Adriaansen-Tennekes, R., Decuypere, E., Parmentier, H.K. & Savelkoul, H.F.J. (2009). Chicken lines selected for their primary antibody response to sheep red blood cells show differential hypothalamic-pituitary-adrenal axis responsiveness to mild stressors. Poultry Science, 88(9), 1879-1882. https://doi.org/10.3382/ps.2009-00150

Al-Nasser, A., Al-Khalaifa, H., Al-Saffar, A., Khalil, F., Albahouh, M., Ragheb, G., ... & Mashaly, M. (2007). Overview of chicken taxonomy and domestication. World's Poultry Science Journal, 63(2), 285-300. https://doi.org/10.1017/S004393390700147X

Al-Rawi, B., & Craig, J. V. (1975). Agonistic behaviour of caged chickens related to group size and area per bird. Applied Animal Ethology, 2(1), 69-80. https://doi.org/10.1016/0304-3762(75)90066-8

Al-Rawi, B., Craig, J. V., & Adams, A. W. (1976). Agonistic behaviour and egg production of caged layers: genetic strain and group-size effects. Poultry Science, 55(2), 796-807. https://doi.org/10.3382/ps.0550796

Amico, J. A., Mantella, R. C., Vollmer, R. R., & Li, X. (2004). Anxiety and stress responses in female oxytocin deficient mice.

Journal of neuroendocrinology, 16(4), 319-324. https://doi.org/10.1111/j.0953-8194.2004.01161.x

Antal, T., Ohtsuki, H., Wakeley, J., Taylor, P. D., & Nowak, M. A. (2009). Evolution of cooperation by phenotypic similarity. Proceedings of the National Academy of Sciences, 106(21), 8597-8600. https://doi.org/10.1073/pnas.0902528106

Appleby, M. C., & Duncan, I. H. (1989). Development of perching in hens. Biology of behaviour (Paris), 14(2), 157-168.

Appleby, M. C., Hughes, B. O., & Elson, H. A. (1992). Poultry production systems. Behaviour, management and welfare. CAB international.

Appleby, M. C. (2003). The European Union ban on conventional cages for laying hens: History and prospects. Journal of applied animal welfare science, 6(2), 103-121. https://doi.org/10.1207/S15327604JAWS0602_03

Appleby, M. C., Mench, J. A., & Hughes, B. O. (2004). Poultry behaviour and welfare. Cabi.

Arnould, C., & Faure, J. M. (2003). Use of pen space and activity of broiler chickens reared at two different densities. Applied Animal Behaviour Science, 84(4), 281-296. https://doi.org/10.1016/j.applanim.2003.07.003

Averós, X., Lorea, A., de Heredia, I. B., Ruiz, R., Marchewka, J., Arranz, J., & Estevez, I. (2014). The behaviour of gestating dairy ewes under different space allowances. Applied Animal Behaviour Science, 150, 17-26. https://doi.org/10.1016/j.applanim.2013.11.002

B

Baarendse, P. J. J., Debonne, M., Decuypere, E., Kemp, B., & Van Den Brand, H. (2007). Ontogeny of avian thermoregulation from a neural point of view. World's Poultry Science Journal, 63(2), 267-276. https://doi.org/10.1017/S0043933907001456

Bailie, C. L., Ball, M. E. E., & O'Connell, N. E. (2013). Influence of the provision of natural light and straw bales on activity levels and leg health in commercial broiler chickens. Animal: an international journal of animal bioscience, 7(4), 618. doi:10.1017/S1751731112002108 PMID: 23171519

Barnard, C. (1991). Kinship and social behaviour: the trouble with relatives. Trends in Ecology & Evolution, 6(10), 310-312.https://doi.org/10.1016/0169-5347(91)90035-V

Barnett, J. L., & Hemsworth, P. H. (1990). The validity of physiological and behavioural measures of animal welfare. Applied Animal Behaviour Science, 25(1-2), 177-187. https://doi.org/10.1016/0168-1591(90)90079-S

Bateson, P. (1979). How do sensitive periods arise and what are they for? Animal Behaviour, 27, 470-486. https://doi.org/10.1016/0003-3472(79)90184-2

Bateson, P. (1990). Is imprinting such a special case? Philosophical Transactions of the Royal Society of London. Series B: Biological Sciences, 329(1253), 125-131. https://doi.org/10.1098/rstb.1990.0157

Bateson, P., Gluckman, P., & Hanson, M. (2014). The biology of developmental plasticity and the Predictive Adaptive Response hypothesis. The Journal of physiology, 592(11), 2357-2368. https://doi.org/10.1113/jphysiol.2014.271460

Belthoff, J. R., & Gauthreaux, S. A. (1991). Aggression and dominance in house finches. The Condor, 93(4), 1010-1013. DOI: 10.2307/3247737 https://www.jstor.org/stable/3247737

Benaroya-Milshtein, N., Hollander, N., Apter, A., Kukulansky, T., Raz, N., Wilf, A., ... & Pick, C. G. (2004). Environmental enrichment in mice decreases anxiety, attenuates stress responses and enhances natural killer cell activity. European Journal of Neuroscience, 20(5), 1341-1347. https://doi.org/10.1111/j.1460-9568.2004.03587.x

Bilcik, B. & Keeling, L. J. (1999). Changing in feather condition in relation to feather pecking and aggressive behaviour in laying hens. British Poultry Science 40(4): 444—451. https://doi.org/10.1080/00071669987188

Bizeray, D., Estevez, I., Leterrier, C., & Faure, J. M. (2002). Effects of increasing environmental complexity on the physical activity of broiler chickens. Applied Animal Behaviour Science, 79(1), 27-41. https://doi.org/10.1016/S0168-1591(02)00083-7

Blokhuis, H. J. (1984). Rest in poultry. Applied Animal Behaviour Science, 12(3), 289-303. https://doi.org/10.1016/0168-1591(84)90121-7

Blokhuis, H. J. (1986). Feather-pecking in poultry: its relation with ground-pecking. Applied Animal Behaviour Science, 16(1), 63-67. https://doi.org/10.1016/0168-1591(86)90040-7

Boissy, A., Manteuffel, G., Jensen, M. B., Moe, R. O., Spruijt, B., Keeling, L. J., ... & Bakken, M. (2007). Assessment of positive emotions in animals to improve their welfare. Physiology & behaviour, 92(3), 375-397. https://doi.org/10.1016/j.physbeh.2007.02.003

Bolhuis, J. J., & Bateson, P. (1990). The importance of being first: a primacy effect in filial imprinting. Animal Behaviour, 40(3), 472-483. https://doi.org/10.1016/S0003-3472(05)80527-5

Bolhuis, J. J., & Honey, R. C. (1998). Imprinting, learning and development: from behaviour to brain and back. Trends in Neurosciences, 21(7), 306-311. https://doi.org/10.1016/S0166-2236(98)01258-2

Bovans Robust- Swedfarm. Recuperado de: http://www.swedfarm.se/index.php/djur/bovans-robust, 2014.

Bradshaw, R. H. (1991). Discrimination of group members by laying hens Gallus domesticus. Behavioural processes, 24(2), 143-151. https://doi.org/10.1016/0376-6357(91)90006-L

Bradshaw, R. H. (1992). Conspecific discrimination and social preference in the laying hen. Applied Animal Behaviour Science, 33(1), 69-75. https://doi.org/10.1016/S0168-1591(05)80086-3

Brake, J., Keeley, T. P., & Jones, R. B. (1994). Effect of age and presence of perches during rearing on tonic immobility fear reactions of broiler breeder pullets. Poultry science, 73(9), 1470-1474. https://doi.org/10.3382/ps.0731470

Brambell, F. W. R. (1965). Report of the technical committee to enquire into the welfare of animals kept under intensive livestock husbandry systems. Her Majesty‟s Stationery Office, London, UK

Broom, D. M. (1986). Indicators of poor welfare. British veterinary journal, 142(6), 524-526.

Broom, D. M. (1991). Animal welfare: concepts and measurement. Journal of animal science, 69(10), 4167-4175. https://doi.org/10.2527/1991.69104167x

Broom, D.M. (2001). Coping, stress and welfare. In Coping with Challenge: Welfare in Animals including Humans, Proceedings of Dahlem Conference, ed. D.M. Broom, 1-9. Berlin: Dahlem University Press

Buitenhuis, B., Hedegaard, J., Janss, L., & Sørensen, P. (2009). Differentially expressed genes for aggressive pecking behaviour in laying hens. BMC genomics, 10(1), 1-10. https://doi.org/10.1186/1471-2164-10-544

C

Campderrich, I., Liste, G., & Estevez, I. (2017). Group size and phenotypic appearance: Their role on the social dynamics in pullets. Applied Animal Behaviour Science, 189, 41-48. https://doi.org/10.1016/j.applanim.2017.01.014

Campo, J. L., & Davila, S. G. (2002). Effect of photoperiod on heterophil to lymphocyte ratio and tonic immobility duration of chickens. Poultry science, 81(11), 1637-1639. https://doi.org/10.1093/ps/81.11.1637

Campo, J. L., Gil, M. G., & Davila, S. G. (2005). Effects of specific noise and music stimuli on stress and fear levels of laying hens of several breeds. Applied Animal Behaviour Science, 91(1-2), 75-84. https://doi.org/10.1016/j.applanim.2004.08.028

Campo, J. L., Gil, M. G., Dávila, S. G., & Muñoz, I. (2007). Effect of lighting stress on fluctuating asymmetry, heterophil-to-lymphocyte ratio, and tonic immobility duration in eleven breeds of chickens. Poultry science, 86(1), 37-45. https://doi.org/10.1093/ps/86.1.37

Campo, J. L., Prieto, M. T., & Davila, S. G. (2008). Effects of housing system and cold stress on heterophil-to-lymphocyte ratio, fluctuating asymmetry, and tonic immobility duration of chickens. Poultry Science, 87(4), 621-626. https://doi.org/10.3382/ps.2007-00466

Campo, J. L., & Prieto, M. T. (2010). Fluctuating asymmetry in hens laying brown eggs with shell color abnormalities or internal inclusions. Archiv für Geflügelkunde, 74(2), 126-132.

Cannon, W. B. (1929). Organization for physiological homeostasis. Physiological reviews, 9(3), 399-431. https://doi.org/10.1152/physrev.1929.9.3.399

Carere C, Caramaschi D, Fawcett TW Covariation between personalities and individual differences in coping with stress: Converging evidence and hypotheses. Current Zoology, 2010; 56(6). https://doi.org/10.1093/czoolo/56.6.728

Cheng, H. W., Freire, R., & Pajor, E. A. (2004). Endotoxin stress responses in chickens from different genetic lines. 1. Sickness, behavioral, and physical responses. Poultry science, 83(5), 707-715. https://doi.org/10.1093/ps/83.5.707

Chrousos, G. P., & Gold, P. W. (1992). The concepts of stress and stress system disorders: overview of physical and behavioral homeostasis. Jama, 267(9), 1244-1252. doi:10.1001/jama.1992.03480090092034

Cichoń, M., Chadzińska, M., Książek, A., & Konarzewski, M. (2002). Delayed effects of cold stress on immune response in laboratory mice. Proceedings of the Royal Society of London. Series B: Biological Sciences, 269(1499), 1493-1497. https://doi.org/10.1098/rspb.2002.2054

Cloutier, S., Beaugrand, J. P., & Laguë, P. C. (1995). The effect of prior victory or defeat in the same site as that of subsequent encounter on the determination of dyadic dominance in the domestic hen. Behavioural processes, 34(3), 293-298. https://doi.org/10.1016/0376-6357(95)00006-G

Cloutier, S., Beaugrand, J. P., & Lague, P. C. (1996). The role of individual differences and patterns of resolution in the formation of dominance orders in domestic hen triads. Behavioural Processes, 38(3), 227-239.

Cloutier, S., & Newberry, R. C. (2002). Differences in skeletal and ornamental traits between laying hen cannibals, victims and bystanders. Applied Animal Behaviour Science, 77(2), 115-126. https://doi.org/10.1016/S0168-1591(02)00049-7

Cloutier, S., Newberry, R. C., Honda, K., & Alldredge, J. R. (2002). Cannibalistic behaviour spread by social learning. Animal Behaviour, 63(6), 1153-1162. https://doi.org/10.1006/anbe.2002.3017

Colditz, I. G., & Hine, B. C. (2016). Resilience in farm animals: biology, management, breeding and implications for animal welfare. Animal Production Science, 56(12), 1961-1983. https://doi.org/10.1071/AN15297

Collias, N. E., Collias, E. C., Hunsaker, D., & Minning, L. (1966). Locality fixation, mobility and social organization within an unconfined population of red jungle fowl. Animal Behaviour, 14(4), 550-559. https://doi.org/10.1016/S0003-3472(66)80059-3

Collias, N. E., & Collias, E. C. (1967). A field study of the red jungle fowl in north-central India. The Condor, 69(4), 360-386. DOI: 10.2307/1366199 https://www.jstor.org/stable/1366199

Collias, N. E., & Collias, E. C. (1996). Social organization of a red junglefowl, Gallus gallus, population related to evolution theory. Animal Behaviour, 51(6), 1337-1354. https://doi.org/10.1006/anbe.1996.0137

Cornetto, T., & Estevez, I. (2001)a. Influence of vertical panels on use of space by domestic fowl. Applied Animal Behaviour Science, 71(2), 141-153. https://doi.org/10.1016/S0168-1591(00)00171-4

Cornetto, T., & Estevez, I. (2001)b. Behavior of the domestic fowl in the presence of vertical panels. Poultry Science, 80(10), 1455-1462. https://doi.org/10.1093/ps/80.10.1455

Cornetto, T., Estevez, I., & Douglass, L. W. (2002). Using artificial cover to reduce aggression and disturbances in domestic fowl. Applied Animal Behaviour Science, 75(4), 325-336. https://doi.org/10.1016/S0168-1591(01)00195-2

Craig, J. V., Biswas, D. K., & Guhl, A. M. (1969). Agonistic behaviour influenced by strangeness, crowding and heredity in female domestic fowl (Gallus gallus). Animal Behaviour, 17, 498-506. https://doi.org/10.1016/0003-3472(69)90153-5

D

Davison, F. (2014). The importance of the avian immune system and its unique features. In Avian immunology (pp. 1-9). Academic Press. https://doi.org/10.1016/B978-0-12-396965-1.00001-7

Dawkins, M. S. (1982). Elusive concept of preferred group size in domestic hens. Applied Animal Ethology, 8(4), 365-375. https://doi.org/10.1016/0304-3762(82)90069-4

Dawkins, M. S. (1983). Battery hens name their price: consumer demand theory and the measurement of ethological ‘needs’. Animal Behaviour, 31(4), 1195-1205.

Dawkins, M. S. (1995). How do hens view other hens? The use of lateral and binocular visual fields in social recognition. Behaviour, 132(7-8), 591-606. https://doi.org/10.1163/156853995X00225

D’Eath, R. B. & Keeling, L. J. (2003). Social discrimination and aggression by laying hens in large groups: from peck orders to social tolerance. Applied animal behaviour science, 84(3), 197-212. https://doi.org/10.1016/j.applanim.2003.08.010

D'Eath, R. B., & Stone, R. J. (1999). Chickens use visual cues in social discrimination: an experiment with coloured lighting. Applied Animal Behaviour Science, 62(2-3), 233-242. https://doi.org/10.1016/S0168-1591(98)00216-0

de Haas, E. N., Bolhuis, J. E., Kemp, B., Groothuis, T. G., & Rodenburg, T. B. (2014). Parents and early life environment affect behavioral development of laying hen chickens. PloS one, 9(3), e90577. https://doi.org/10.1371/journal.pone.0090577

Dennis, R. L., Newberry, R. C., Cheng, H. W., & Estevez, I. (2008). Appearance matters: artificial marking alters aggression and stress. Poultry science, 87(10), 1939-1946. https://doi.org/10.3382/ps.2007-00311

Dey, C. J., Dale, J., & Quinn, J. S. (2014). Manipulating the appearance of a badge of status causes changes in true badge expression. Proceedings of the Royal Society B: Biological Sciences, 281(1775), 20132680. https://doi.org/10.1098/rspb.2013.2680 pmid:24285201

Dhabhar, F. S., & McEwen, B. S. (1997). Acute stress enhances while chronic stress suppresses cell-mediated immunityin vivo: A potential role for leukocyte trafficking. Brain, behaviour, and immunity, 11(4), 286-306. https://doi.org/10.1006/brbi.1997.0508

Dhabhar, F. S., Miller, A. H., McEwen, B. S., & Spencer, R. L. (1995). Effects of stress on immune cell distribution. Dynamics and hormonal mechanisms. The Journal of Immunology, 154(10), 5511-5527.

Dhabhar, F. S., Miller, A. H., McEwen, B. S., & Spencer, R. L. (1996). Stress-induced changes in blood leukocyte distribution. Role of adrenal steroid hormones. The journal of immunology, 157(4), 1638-1644.

Di Giorgio, E., Loveland, J. L., Mayer, U., Rosa-Salva, O., Versace, E., & Vallortigara, G. (2017). Filial responses as predisposed and learned preferences: Early attachment in chicks and babies. Behavioural brain research, 325, 90-104. https://doi.org/10.1016/j.bbr.2016.09.018

Dohms, J. E., & Metz, A. (1991). Stress—mechanisms of immunosuppression. Veterinary Immunology and Immunopathology, 30(1), 89-109. https://doi.org/10.1016/0165-2427(91)90011-Z

Dunbar, R. I. (1991). Functional significance of social grooming in primates. Folia primatologica, 57(3), 121-131. DOI: 10.1159/000156574

Dunbar, R. I. (1992). Neocortex size as a constraint on group size in primates. Journal of human evolution, 22(6), 469-493.

E

Eklund, B., & Jensen, P. (2011). Domestication effects on behavioural synchronization and individual distances in chickens (Gallus gallus). Behavioural processes, 86(2), 250-256. DOI: 10.1016/j.beproc.2010.12.010

Elton, C. (1939). On the nature of cover. The Journal of Wildlife Management, 3(4), 332-338. DOI: 10.2307/3796305

Ericsson, M. (2016). Stress in chickens: Effects of domestication and early experience on behaviour and welfare (Doctoral dissertation, Linköping University Electronic Press). DOI: 10.3384/diss.diva-126236

Ericsson, M., Henriksen, R., Bélteky, J., Sundman, A. S., Shionoya, K., & Jensen, P. (2016). Long-term and transgenerational effects of stress experienced during different life phases in chickens (Gallus gallus). PLoS One, 11(4), e0153879. DOI: 10.1371/journal.pone.0153879

Eriksson, J., Larson, G., Gunnarsson, U., Bed'Hom, B., Tixier-Boichard, M., Strömstedt, L., ... & Jensen, P. (2008). Identification of the yellow skin gene reveals a hybrid origin of the domestic chicken. PLoS Genet, 4(2), e1000010. https://doi.org/10.1371/journal.pgen.1000010

Estevez, I., Newberry, R. C., & De Reyna, L. A. (1997). Broiler chickens: a tolerant social system. Etologia, 5(1).

Estevez, I., Newberry, R. C., & Keeling, L. J. (2002). Dynamics of aggression in the domestic fowl. Applied Animal Behaviour Science, 76(4), 307-325. https://doi.org/10.1016/S0168-1591(02)00013-8

Estevez, I., Keeling, L. J., & Newberry, R. C. (2003). Decreasing aggression with increasing group size in young domestic fowl. Applied Animal Behaviour Science, 84(3), 213-218. https://doi.org/10.1016/j.applanim.2003.08.006

Estevez, I., Andersen, I. L., & Nævdal, E. (2007). Group size, density and social dynamics in farm animals. Applied Animal Behaviour Science, 103(3-4), 185-204. https://doi.org/10.1016/j.applanim.2006.05.025

F

Farm Animal Welfare Council (1993). Second Report on Priorities for Research and Development in Farm Animal welfare. MAFF Publ., Tolworth, London, UK.

Fraser, D. (2004, February). Applying science to animal welfare standards. In Proceedings of Global conference on Animal Welfare: an OIE initiative (pp. 23-25). Paris: OIE.

Fraser, A. F., & Broom, D. M. (1997). Farm animal behaviour and welfare (No. Ed. 3). CAB international.

Frölich, E. K. F., Niebuhr, K., Schrader, L., Oester, H. (2012), 'What are alternative systems for poultry?'. in V Sandilands & PM Hocking (eds), Alternative systems for poultry: health, welfare and productivity. Poultry Science Symposium Series, vol. 30, CABI International, Wallingford, pp. 340-348.

Fumihito, A., Miyake, T., Takada, M., Shingu, R., Endo, T., Gojobori, T., ... & Ohno, S. (1996). Monophyletic origin and unique dispersal patterns of domestic fowls. Proceedings of the National Academy of Sciences, 93(13), 6792-6795. https://doi.org/10.1073/pnas.93.13.6792

G

García-Navas, V., Ferrer, E. S., & Sanz, J. J. (2013). Prey choice, provisioning behaviour, and effects of early nutrition on nestling phenotype of titmice. Ecoscience, 20(1), 9-18. https://doi.org/10.2980/20-1-3545

Gerlach, G., & Lysiak, N. (2006). Kin recognition and inbreeding avoidance in zebrafish, Danio rerio, is based on phenotype matching. Animal Behaviour, 71(6), 1371-1377. https://doi.org/10.1016/j.anbehav.2005.10.010

Ghazi, S. H., Habibian, M., Moeini, M. M., & Abdolmohammadi, A. R. (2012). Effects of different levels of organic and inorganic chromium on growth performance and immunocompetence of broilers under heat stress. Biological trace element research, 146(3), 309-317. https://doi.org/10.1007/s12011-011-9260-1

Göerlich, V. C., Nätt, D., Elfwing, M., Macdonald, B., & Jensen, P. (2012). Transgenerational effects of early experience on behavioral, hormonal and gene expression responses to acute stress in the precocial chicken. Hormones and behaviour, 61(5), 711-718. https://doi.org/10.1016/j.yhbeh.2012.03.006

Goodall, J. (1986). The chimpanzees of Gombe: Patterns of behaviour. Cambridge Mass.

Grafen, A. (1990). Do animals really recognize kin? Animal Behaviour, 39(1), 42-54. https://doi.org/10.1016/S0003-3472(05)80724-9

Guhl, A. M. (1968). Social behaviour of the domestic fowl. Transactions of the Kansas Academy of Science (1903-), 71(3), 379-384. DOI: 10.2307/3627156 pmid:5704248

Guhl, A. M., & Allee, W. C. (1944). Some measurable effects of social organization in flocks of hens. Physiological Zoology, 17(3), 320-347.

Guhl, A. M., & Ortman, L. L. (1953). Visual patterns in the recognition of individuals among chickens. The Condor, 55(6), 287-298. DOI: 10.2307/1365008

Gunnarsson, S., Yngvesson, J., Keeling, L. J., & Forkman, B. (2000). Rearing without early access to perches impairs the spatial skills of laying hens. Applied Animal Behaviour Science, 67(3), 217-228. https://doi.org/10.1016/S0168-1591(99)00125-2

H

Hamilton, W.D. (1964)a. The genetic evolution of social behaviour. I. Journal of theoretical biology, 7(1), 1–17.

Hamilton, W.D. (1964)b. The genetic evolution of social behaviour. II. Journal of theoretical biology, 7(1), 17-52.

Hamilton, W. D., & Zuk, M. (1982). Heritable true fitness and bright birds: a role for parasites? Science, 218(4570), 384-387. DOI: 10.1126/science.7123238 pmid:7123238

Harrison, C. J. (1965). Allopreening as agonistic behaviour. Behaviour, 24(3-4), 161-208. https://doi.org/10.1163/156853965X00011

Harrison, R. (1964). Animal Machines: The New Factory Farming Industry. London: Vincent Stuart Ltd. https://doi.org/10.1163/156853965X00011

Hauser, J., & Huber-Eicher, B. (2004). Do domestic hens discriminate between familiar and unfamiliar conspecifics in the absence of visual cues? Applied Animal Behaviour Science, 85(1-2), 65-76. https://doi.org/10.1016/j.applanim.2003.09.006

Haye, U., & Simons, P. C. M. (1978). Twisted legs in broilers. British Poultry Science, 19(4), 549-557. https://doi.org/10.1080/00071667808416512

Henzi, S. P., Lycett, J. E., & Piper, S. E. (1997)a. Fission and troop size in a mountain baboon population. Animal Behaviour, 53(3), 525-535. https://doi.org/10.1006/anbe.1996.0302

Henzi, S. P., Lycett, J. E., & Weingrill, T. (1997)b. Cohort size and the allocation of social effort by female mountain baboons.

Animal Behaviour, 54(5), 1235-1243. https://doi.org/10.1006/anbe.1997.0520

Hess, EH (1958). Imprinting in animals. Scientific American, 198, 81-90.

Hocking, P. M., Bain, M., Channing, C. E., Fleming, R., & Wilson, S. (2003). Genetic variation for egg production, egg quality and bone strength in selected and traditional breeds of laying fowl. British poultry science, 44(3), 365-373. https://doi.org/10.1080/0007166031000085535

Hõrak, P., Ots, I., Vellau, H., Spottiswoode, C., & Møller, A. P. (2001). Carotenoid-based plumage coloration reflects hemoparasite infection and local survival in breeding great tits. Oecologia, 126(2), 166-173. https://doi.org/10.1007/s004420000513 pmid:28547614

Huber-Eicher, B., & Audigé, L. (1999). Analysis of risk factors for the occurrence of feather pecking in laying hen growers. British poultry science, 40(5), 599-604. https://doi.org/10.1080/00071669986963

Huff, G. R., Huff, W. E., Balog, J. M., Rath, N. C., Anthony, N. B., & Nestor, K. E. (2005). Stress response differences and disease susceptibility reflected by heterophil to lymphocyte ratio in turkeys selected for increased body weight. Poultry science, 84(5), 709-717.https://doi.org/10.1093/ps/84.5.709

Hughes, B. O., Carmichael, N. L., Walker, A. W., & Grigor, P. N. (1997). Low incidence of aggression in large flocks of laying hens. Applied Animal Behaviour Science, 54(2-3), 215-234. https://doi.org/10.1016/S0168-1591(96)01177-X

Hughes, B. O., & Wood-Gush, D. G. M. (1977). Agonistic behaviour in domestic hens: the influence of housing method and group size. Animal behaviour, 25, 1056-1062. https://doi.org/10.1016/0003-3472(77)90056-2 pmid:564148

Hughes, D. P., & Cremer, S. (2007). Plasticity in antiparasite behaviours and its suggested role in invasion biology. Animal Behaviour, 74(5), 1593-1599.

Hurst, J. L., & Barnard, C. J. (1992). Kinship and social behaviour in wild house mice: effects of social group membership and relatedness on the responses of dominant males toward juveniles. Behavioral Ecology, 3(3), 196-206. https://doi.org/10.1093/beheco/3.3.196

I

J

Jones, R. B., & Faure, J. M. (1982). Domestic chicks prefer familiar soiled substrate in an otherwise novel environment. IRCS Med. Sci, 10, 847.

Jones, R. B., & Gentle, M. J. (1985). Olfaction and behavioral modification in domestic chicks (Gallus domesticus). Physiology & behaviour, 34(6), 917-924. https://doi.org/10.1016/0031-9384(85)90014-9 pmid:4059381

Jones, R. B. (1989). Chronic stressors, tonic immobility and leucocytic responses in the domestic fowl. Physiology & behaviour, 46(3), 439-442. https://doi.org/10.1016/0031-9384(89)90017-6

Jones, M. A., Mason, G., & Pillay, N. (2010). Early social experience influences the development of stereotypic behaviour in captive-born striped mice Rhabdomys. Applied Animal Behaviour Science, 123(1-2), 70-75. https://doi.org/10.1016/j.applanim.2009.12.009

K

Kathleen, M., & Tomborg, C. (2007). Sources of stress in captivity. Applied Animal Behavior Science, 102, 262-302. https://doi.org/10.1016/j.applanim.2006.05.032.

Keeling, L. J. (1997, August). A comparison of two basic characteristics of a perch for laying hens. In Proceedings of the 31st International Congress of the ISAE, Research Institute of Animal Production (pp. 13-16).

Keeling, L. J., Gonyou, H. W. Eds., (2001). Social Behaviour in Farm Animals. CABI Publishing, New York.

Keeling, L. J., Estevez, I., Newberry, R. C., & Correia, M. G. (2003). Production-related traits of layers reared in different sized flocks: the concept of problematic intermediate group sizes. Poultry science, 82(9), 1393-1396. https://doi.org/10.1093/ps/82.9.1393

Keeling, L., Andersson, L., Schütz, K. E., Kerje, S., Fredriksson, R., Carlborg, Ö., ... & Jensen, P. (2004). Feather-pecking and victim pigmentation. Nature, 431(7009), 645-646. https://doi.org/10.1038/431645a

Komdeur, J., & Hatchwell, B. J. (1999). Kin recognition: function and mechanism in avian societies. Trends in Ecology &

Evolution, 14(6), 237-241. https://doi.org/10.1016/S0169-5347(98)01573-0

Koolhaas, J. M., Bartolomucci, A., Buwalda, B., de Boer, S. F., Flügge, G., Korte, S. M., ... & Richter-Levin, G. (2011). Stress revisited: a critical evaluation of the stress concept. Neuroscience & Biobehavioral Reviews, 35(5), 1291-1301. https://doi.org/10.1016/j.neubiorev.2011.02.003

L

Lara, L. J., & Rostagno, M. H. (2013). Impact of heat stress on poultry production. Animals, 3(2), 356-369. https://doi.org/10.3390/ani3020356

Lay Jr, D. C., Fulton, R. M., Hester, P. Y., Karcher, D. M., Kjaer, J. B., Mench, J. A., ... & Porter, R. E. (2011). Hen welfare in different housing systems. Poultry science, 90(1), 278-294. https://doi.org/10.3382/ps.2010-00962

Lechner, O., Dietrich, H., Wiegers, G. J., Vacchio, M., & Wick, G. (2001). Glucocorticoid production in the chicken bursa and thymus. International Immunology, 13(6), 769-776. https://doi.org/10.1093/intimm/13.6.769

Lee, K. A. (2006). Linking immune defenses and life history at the levels of the individual and the species. Integrative and comparative biology, 46(6), 1000-1015. https://doi.org/10.1093/icb/icl049

Leenstra, F., Maurer, V., Bestman, M., van Sambeek, F., Zeltner, E., Reuvekamp, B., ... & van Niekerk, T. (2012). Performance of commercial laying hen genotypes on free range and organic farms in Switzerland, France and The Netherlands. British poultry science, 53(3), 282-290. https://doi.org/10.1080/00071668.2012.703774

Lehmann, J., Korstjens, A. H., & Dunbar, R. I. M. (2007). Group size, grooming and social cohesion in primates. Animal Behaviour, 74(6), 1617-1629. https://doi.org/10.1016/j.anbehav.2006.10.025

Leinonen, I., Williams, A. G., Wiseman, J., Guy, J., & Kyriazakis, I. (2012). Predicting the environmental impacts of chicken systems in the United Kingdom through a life cycle assessment: Broiler production systems. Poultry science, 91(1), 8-25. https://doi.org/10.3382/ps.2011-01634

Leone, E. H., Estevez, I., & Christman, M. C. (2007). Environmental complexity and group size: Immediate effects on use of space by domestic fowl. Applied Animal Behaviour Science, 102(1-2), 39-52. https://doi.org/10.1016/j.applanim.2006.03.004

Leone, E. H., & Estevez, I. (2008). Use of space in the domestic fowl: separating the effects of enclosure size, group size and density. Animal Behaviour, 76(5), 1673-1682. https://doi.org/10.1016/j.anbehav.2008.08.004

Lewis, S., Roberts, G., Harris, M. P., Prigmore, C., & Wanless, S. (2007). Fitness increases with partner and neighbour allopreening. Biology letters, 3(4), 386-389. https://doi.org/10.1098/rsbl.2007.0258

Lewis, K. (1998). Pathogen resistance as the origin of kin altruism. Journal of Theoretical Biology, 193(2), 359-363. https://doi.org/10.1006/jtbi.1998.0725

Leyendecker, M., Hamann, H., Hartung, J., Kamphues, J., Neumann, U., Sürie, C., & Distl, O. (2005). Keeping laying hens in furnished cages and an aviary housing system enhances their bone stability. British Poultry Science, 46(5), 536-544. https://doi.org/10.1080/00071660500273094

Lindqvist, C., Janczak, A. M., Nätt, D., Baranowska, I., Lindqvist, N., Wichman, A., ... & Jensen, P. (2007). Transmission of stress-induced learning impairment and associated brain gene expression from parents to offspring in chickens. PloS one, 2(4), e364. https://doi.org/10.1371/journal.pone.0000364

Liste, G., Campderrich, I., de Heredia, I. B., & Estevez, I. (2015). The relevance of variations in group size and phenotypic appearance on the behaviour and movement patterns of young domestic fowl. Applied Animal Behaviour Science, 163, 144-157. https://doi.org/10.1016/j.applanim.2014.11.013

Littell, R. C., Milliken, G. A., Stroup, W. W., Wolfinger, R. D., & Oliver, S. (2006). SAS for mixed models. SAS publishing.

Lizé, A., Carval, D., Cortesero, A. M., Fournet, S., & Poinsot, D. (2006). Kin discrimination and altruism in the larvae of a solitary insect. Proceedings of the Royal Society B: Biological Sciences, 273(1599), 2381-2386. https://doi.org/10.1098/rspb.2006.3598

Lorenz, K. (1935). Der Kumpan in der Umwelt des Vogels. Der Artgenosse als auslösendes Moment sozialer Verhaltensweisen. *Journal für Ornithologie, 83:* 137–215, 289–413. https://doi.org/10.1007/BF01905355

Lorenz, K. (1937). Imprinting. Auk, 54(1), 245-273.

M

Maier, R. A. (1964). The role of the dominance-submission ritual in social recognition of hens. Animal Behaviour, 12(1), 59.

Malleau AE, Duncan IJ, Widowski TM, Atkinson JL. The importance of rest in young domestic fowl. Applied Animal Behaviour Science, 2007; 106(1): 52-69. https://doi.org/10.1016/j.applanim.2006.06.017

Manteca, X. (2015). Animal welfare: Concept and importance. Zoo Animal Welfare. Concept and Indicators, 1st ed.; Multimédica Ediciones Veterinarias: Sant Cugat del Vallés, Barcelona, Spain, 1-25.

Marin, R. H., Freytes, P., Guzman, D., & Jones, R. B. (2001). Effects of an acute stressor on fear and on the social reinstatement responses of domestic chicks to cagemates and strangers. Applied Animal Behaviour Science, 71(1), 57-66. doi: 10.1016/s0168-1591(00)00167-2.

Marin, R. H., Liste, M. G., Campderrich, I., & Estevez, I. (2014). The impact of phenotypic appearance on body weight and egg production in laying hens: a group-size-and experience-dependent phenomenon. Poultry science, 93(7), 1623-1635. https://doi.org/10.3382/ps.2013-03705

Marks, H. L., Siegel, P. B., & Kramer, C. Y. (1960). Effect of comb and wattle removal on the social organization of mixed flocks of chickens. Animal Behaviour. https://doi.org/10.1016/0003-3472(60)90026-9

Martin, F., Beaugrand, J. P., & Laguë, P. C. (1997). The role of recent experience and weight on hen's agonistic behaviour during dyadic conflict resolution. Behavioural Processes, 41(2), 159-170. https://doi.org/10.1016/S0376-6357(97)00044-2

Mashaly, M. M., Hendricks 3rd, G. L., Kalama, M. A., Gehad, A. E., Abbas, A. O., & Patterson, P. H. (2004). Effect of heat stress on production parameters and immune responses of commercial laying hens. Poultry science, 83(6), 889-894. https://doi.org/10.1093/ps/83.6.889

Mateo, J. M., & Johnston, R. E. (2000). Kin recognition and the ‘armpit effect’: evidence of self–referent phenotype matching. Proceedings of the Royal Society of London. Series B: Biological Sciences, 267(1444), 695-700. https://doi.org/10.1098/rspb.2000.1058

McAdie, T. M., & Keeling, L. J. (2000). Effect of manipulating feathers of laying hens on the incidence of feather pecking and cannibalism. Applied Animal Behaviour Science, 68(3), 215-229. https://doi.org/10.1016/S0168-1591(00)00107-6

McBride, G. (1960). Poultry husbandry and the peck order. British Poultry Science, 1(1-3), 65-68. https://doi.org/10.1080/00071666009382378

McBride, G., & Foenander, F. (1962). Territorial behaviour in flocks of domestic fowls. Nature, 194(4823), 102-102. https://doi.org/10.1038/194102a0

McBride, G., Parer, I. P., & Foenander, F. (1969). The social organization and behaviour of the feral domestic fowl. Animal Behaviour Monographs, 2, 125-181. https://doi.org/10.1016/S0066-1856(69)80003-8

Mellor, D. J. (2015). Enhancing animal welfare by creating opportunities for positive affective engagement. New Zealand Veterinary Journal, 63(1), 3-8. https://doi.org/10.1080/00480169.2014.926799

Mellor, D. J. (2016). Updating animal welfare thinking: Moving beyond the "Five Freedoms" towards "a Life Worth Living". Animals, 6(3), 21. https://doi.org/10.3390/ani6030021

Mench, J., & Keeling, L. J. (2001). The social behaviour of domestic birds. Social behaviour in farm animals, 177-211.

Moberg, G. P. (2000). Biological response to stress: implications for animal welfare. The biology of animal stress: basic principles and implications for animal welfare, 1, 21.

Moberg, G. P., & Mench, J. A. (Eds.). (2000). The biology of animal stress: basic principles and implications for animal welfare. CABI.

Mokkonen, M., & Lindstedt, C. (2016). The evolutionary ecology of deception. Biological Reviews, 91(4), 1020-1035. https://doi.org/10.1111/brv.12208

Møller, A. P., Sanotra, G. S., & Vestergaard, K. S. (1995). Developmental stability in relation to population density and breed of chickens Gallus gallus. Poultry Science, 74(11), 1761-1771. https://doi.org/10.3382/ps.0741761

Mujahid, A., & Furuse, M. (2009). Behavioral responses of neonatal chicks exposed to low environmental temperature. Poultry Science, 88(5), 917-922. https://doi.org/10.3382/ps.2008-00472

Murray, D. L., & Fuller, M. R. (2000). A critical review of the effects of marking on the biology of vertebrates. Research techniques in animal ecology: controversies and consequences, 15-64.

Muñoz-Abellán, C., Rabasa, C., Daviu, N., Nadal, R., & Armario, A. (2011). Behavioral and endocrine consequences of simultaneous exposure to two different stressors in rats: interaction or independence?. PLoS One, 6(6), https://doi.org/10.1371/journal.pone.0021426

N

Nazar, F. N., & Marin, R. H. (2011). Chronic stress and environmental enrichment as opposite factors affecting the immune response in Japanese quail (Coturnix coturnix japonica). Stress, 14(2), 166-173. https://doi.org/10.3109/10253890.2010.523093

Nazar, F. N., Marin, R. H., Liste, G., Campderrich, I., & Estevez, I. (2015). Manipulation of the phenotypic appearance of individuals in groups of laying hens: effects on stress and immune-related variables. Stress, 18(6), 710-717. https://doi.org/10.3109/10253890.2015.1078306

Nelson, J. R., McIntyre, D. R., Pavlidis, H. O., & Archer, G. S. (2018). Reducing stress susceptibility of broiler chickens by supplementing a yeast fermentation product in the feed or drinking water. Animals, 8(10), 173. https://doi.org/10.3390/ani8100173

Nettle, D., & Bateson, M. (2015). Adaptive developmental plasticity: what is it, how can we recognize it and when can it evolve?. Proceedings of the Royal Society B: Biological Sciences, 282(1812), https://doi.org/10.1098/rspb.2015.1005

Neumann, I. D., Wigger, A., Torner, L., Holsboer, F., & Landgraf, R. (2000). Brain Oxytocin Inhibits Basal and Stress-Induced Activity of the Hypothalamo-Pituitary-Adrenal Axis in Male and Female Rats: Partial Action Within the Paraventricular Nucleus. Journal of Neuroendocrinology, 12(3), 235–243. https://doi.org/10.1046/j.1365-2826.2000.00442.x

Neumann, I. D. (2008). Brain oxytocin: a key regulator of emotional and social behaviours in both females and males. Journal of neuroendocrinology, 20(6), 858-865. https://doi.org/10.1111/j.1365-2826.2008.01726.x

Newberry, R. C. (1995). Environmental enrichment: increasing the biological relevance of captive environments. Applied Animal Behaviour Science, 44(2-4), 229-243.

Newberry, R. C., & Hall, J. W. (1990). Use of pen space by broiler chickens: effects of age and pen size. Applied Animal Behaviour

Science, 25(1-2), 125-136. https://doi.org/10.1016/0168-1591(90)90075-O

Newberry, R. C., & Shackleton, D. M. (1997). Use of visual cover by domestic fowl: a Venetian blind effect?. Animal Behaviour, 54(2), 387-395. https://doi.org/10.1006/anbe.1996.0421

Newberry, R., & Cloutier, S. (2000). Recent social experience, body weight and initial patterns of attack predict the social status attained by unfamiliar hens in a new group. Behaviour, 137(6), 705-726. https://doi.org/10.1163/156853900502303

Newberry, R. C., Estevez, I., & Keeling, L. J. (2001). Group size and perching behaviour in young domestic fowl. Applied Animal Behaviour Science, 73(2), 117-129. https://doi.org/10.1016/S0168-1591(01)00135-6

Newberry, R. C., Keeling, L. J., Estevez, I., & Bilčík, B. (2007). Behaviour when young as a predictor of severe feather pecking in adult laying hens: the redirected foraging hypothesis revisited. Applied Animal Behaviour Science, 107(3-4), 262-274. https://doi.org/10.1016/j.applanim.2006.10.010

Newman, S., & Leeson, S. (1998). Effect of housing birds in cages or an aviary system on bone characteristics. Poultry science, 77(10), 1492-1496. https://doi.org/10.1093/ps/77.10.1492

Nicol, C. J., Gregory, N. G., Knowles, T. G., Parkman, I. D., & Wilkins, L. J. (1999). Differential effects of increased stocking density, mediated by increased flock size, on feather pecking and aggression in laying hens. Applied Animal Behaviour Science, 65(2), 137-152. https://doi.org/10.1016/S0168-1591(99)00057-X

O

O’Connor, E. A., Saunders, J. E., Grist, H., McLeman, M. A., Wathes, C. M., & Abeyesinghe, S. M. (2011). The relationship between the comb and social behaviour in laying hens. Applied Animal Behaviour Science, 135(4), 293-299. https://doi.org/10.1016/j.applanim.2011.09.011

Odén, K., Keeling, L. J., & Algers, B. (2002). Behaviour of laying hens in two types of aviary systems on 25 commercial farms in Sweden. British poultry science, 43(2), 169-181. https://doi.org/10.1080/00071660120121364

Olfati, A., Mojtahedin, A., Sadeghi, T., Akbari, M., & Martínez-Pastor, F. (2018). Comparison of growth performance and immune responses of broiler chicks reared under heat stress, cold stress and thermoneutral conditions. Spanish Journal of Agricultural Research, 16(2), 15.

O'Mahony, S. M., Marchesi, J. R., Scully, P., Codling, C., Ceolho, A. M., Quigley, E. M., ... & Dinan, T. G. (2009). Early life stress alters behaviour, immunity, and microbiota in rats: implications for irritable bowel syndrome and psychiatric illnesses. Biological psychiatry, 65(3), 263-267. https://doi.org/10.1016/j.biopsych.2008.06.026

P

Pagel, M., & Dawkins, M. S. (1997). Peck orders and group size in laying hens: futures contracts' for non-aggression. Behavioural Processes, 40(1), 13-25. https://doi.org/10.1016/S0376-6357(96)00761-9

Piersma, T., & Drent, J. (2003). Phenotypic flexibility and the evolution of organismal design. Trends in Ecology & Evolution, 18(5), 228-233. https://doi.org/10.1016/S0169-5347(03)00036-3

Price, E. O. (1999). Behavioral development in animals undergoing domestication. Applied Animal Behaviour Science, 65(3), 245-271. https://doi.org/10.1016/S0168-1591(99)00087-8

Q

Queiroz, S. A., & Cromberg, V. U. (2006). Aggressive behaviour in the genus Gallus sp. Brazilian Journal of Poultry Science, 8(1), 1-14. https://doi.org/10.1590/S1516-635X2006000100001

Quinteiro-Filho, W. M., Rodrigues, M. V., Ribeiro, A., Ferraz-de-Paula, V., Pinheiro, M. L., Sá, L., ... & Palermo-Neto, J. (2012). Acute heat stress impairs performance parameters and induces mild intestinal enteritis in broiler chickens: Role of acute hypothalamic-pituitary-adrenal axis activation. Journal of animal science, 90(6), 1986-1994. https://doi.org/10.2527/jas.2011-3949

R

Real Decreto 1201, 2005. Sobre protección de los animales utilizados para experimentación y otros fines científicos., pp. 34367–34391, BOE núm.252.21/10/2005.

Riedstra, B., & Groothuis, T. G. (2002). Early feather pecking as a form of social exploration: the effect of group stability on feather pecking and tonic immobility in domestic chicks. Applied Animal Behaviour Science, 77(2), 127-138. https://doi.org/10.1016/S0168-1591(02)00031-X

Roberts, M. L., Buchanan, K. L., Evans, M. R., Marin, R. H., & Satterlee, D. G. (2009). The effects of testosterone on immune function in quail selected for divergent plasma corticosterone response. Journal of Experimental Biology, 212(19), 3125-3131. https://doi.org/10.1242/jeb.030726

Rodenburg, T. B., & Koene, P. (2007). The impact of group size on damaging behaviours, aggression, fear and stress in farm animals. Applied Animal Behaviour Science, 103(3-4), 205-214. https://doi.org/10.1016/j.applanim.2006.05.024

Rodenburg, T. B., Komen, H., Ellen, E. D., Uitdehaag, K. A., & van Arendonk, J. A. (2008). Selection method and early-life history affect behavioural development, feather pecking and cannibalism in laying hens: a review. Applied Animal Behaviour Science, 110(3-4), 217-228. https://doi.org/10.1016/j.applanim.2007.09.009

Rodríguez, S., Van Noordwijk, A. J., Álvarez, E., & Barba, E. (2016). A recipe for postfledging survival in great tits Parus major: be large and be early (but not too much). Ecology and evolution, 6(13), 4458-4467. https://doi.org/10.1002/ece3.2192

Rodriguez-Aurrekoetxea, A., Leone, E. H., & Estevez, I. (2014). Environmental complexity and use of space in slow growing free range chickens. Applied Animal Behaviour Science, 161, 86-94. https://doi.org/10.1016/j.applanim.2014.09.014

Rohwer, S., & Rohwer, F. C. (1978). Status signalling in Harris sparrows: experimental deceptions achieved. Animal behaviour, 26, 1012-1022. https://doi.org/10.1016/0003-3472(78)90090-8

Roll, V. F. B., Levrino, G. A. M., & Briz, R. C. (2008). Rearing system and behavioural adaptation of laying hens to furnished cages. Ciência Rural, 38(7), 1997-2003. http://dx.doi.org/10.1590/S0103-84782008000700031

Romanov, M. N., & Weigend, S. (2001). Analysis of genetic relationships between various populations of domestic and jungle fowl using microsatellite markers. Poultry science, 80(8), 1057-1063. https://doi.org/10.1093/ps/80.8.1057

Romero, M. L., & Butler, L. K. (2007). Endocrinology of stress. International Journal of Comparative Psychology, 20(2). Retrieved from https://escholarship.org/uc/item/87d2k2xz

Rushen, J. (1982). The peck orders of chickens: how do they develop and why are they linear?. Animal Behaviour, 30(4), 1129-1137. https://doi.org/10.1016/S0003-3472(82)80203-0

Rygula, R., Papciak, J., & Popik, P. (2013). Trait pessimism predicts vulnerability to stress-induced anhedonia in rats. Neuropsychopharmacology, 38(11), 2188-2196. https://doi.org/10.1038/npp.2013.116

S

Satterlee, D. G., Cadd, G. G., & Jones, R. B. (2000). Developmental instability in Japanese quail genetically selected for contrasting adrenocortical responsiveness. Poultry Science, 79(12), 1710-1714. https://doi.org/10.1093/ps/79.12.1710

Schjelderup-Ebbe, T. (1922). Contributions to the social psychology of the domestic chicken. Reprinted from Zeitschrift fuer Psychologie, 88, 225-252.

Schrader, L., & Müller, B. (2009). Night-time roosting in the domestic fowl: The height matters. Applied Animal Behaviour Science, 121(3-4), 179-183. https://doi.org/10.1016/j.applanim.2009.09.010

Selye, H. (1946). The general adaptation syndrome and the diseases of adaptation. The journal of clinical endocrinology, 6(2), 117-230. https://doi.org/10.1210/jcem-6-2-117

Selye, H. (1973). The Evolution of the Stress Concept: The originator of the concept traces its development from the discovery in 1936 of the alarm reaction to modern therapeutic applications of syntoxic and catatoxic hormones. American scientist, 61(6), 692-699. https://www.jstor.org/stable/27844072

Senar, J. C., Camerino, M., Copete, J. L., & Metcalfe, N. B. (1993). Variation in black bib of the Eurasian siskin (Carduelis spinus) and its role as a reliable badge of dominance. The Auk, 924-927. https://doi.org/10.2307/4088649

Sever, J. L. (1962). Application of a microtechnique to viral serological investigations. The Journal of Immunology, 88(3), 320-329. PMED: 13910995

Shinder, D., Luger, D., Rusal, M., Rzepakovsky, V., Bresler, V., & Yahav, S. (2002). Early age cold conditioning in broiler chickens (Gallus domesticus): thermotolerance and growth responses. Journal of Thermal Biology, 27(6), 517-523. https://doi.org/10.1016/S0306-4565(02)00025-6

Shini, S., & Kaiser, P. (2009). Effects of stress, mimicked by administration of corticosterone in drinking water, on the expression of chicken cytokine and chemokine genes in lymphocytes. Stress, 12(5), 388-399. https://doi.org/10.1080/10253890802526894

Shini, S., Huff, G. R., Shini, A., & Kaiser, P. (2010). Understanding stress-induced immunosuppression: exploration of cytokine and chemokine gene profiles in chicken peripheral leukocytes. Poultry science, 89(4), 841-851. https://doi.org/10.3382/ps.2009-00483

Siegel, P. B., & Hurst, D. C. (1962). Social interactions among females in dubbed and undubbed flocks. Poultry Science, 41(1), 141-145. https://doi.org/10.3382/ps.0410141

Smits, J. E., & Baos, R. (2005). Evaluation of the antibody mediated immune response in nestling American kestrels (Falco sparverius). Developmental & Comparative Immunology, 29(2), 161-170. https://doi.org/10.1016/j.dci.2004.06.007

Smits, J. E., & Williams, T. D. (1999). Validation of immunotoxicology techniques in passerine chicks exposed to oil sands tailings water. Ecotoxicology and Environmental Safety, 44(1), 105-112. https://doi.org/10.1006/eesa.1999.1806

Sossidou, E. N., & Elson, H. A. (2009). Hens' welfare to egg quality: a European perspective. World's Poultry Science Journal, 65(4), 709-718. https://doi.org/10.1017/S0043933909000488

Sparks, J. H. (1964). Flock structure of the red avadavat with particular reference to clumping and allopreening. Animal

Behaviour, 12(1), 125-136. https://doi.org/10.1016/0003-3472(64)90113-7

Stahl, P., Ruette, S., & Gros, L. (2002). Predation on free-ranging poultry by mammalian and avian predators: field loss estimates in a French rural area. Mammal review, 32(3), 227-234. https://doi.org/10.1046/j.1365-2907.2002.00110.x

St-Pierre, N. R., Cobanov, B., & Schnitkey, G. (2003). Economic losses from heat stress by US livestock industries. Journal of dairy science, 86, E52-E77. https://doi.org/10.3168/jds.S0022-0302(03)74040-5

Struelens, E., Tuyttens, F. A. M., Duchateau, L., Leroy, T., Cox, M., Vranken, E., ... & Sonck, B. (2008). Perching behaviour and perch height preference of laying hens in furnished cages varying in height. British poultry science, 49(4), 381-389. https://doi.org/10.1080/00071660802158332

Suk, Y. O., & Washburn, K. W. (1995). Effects of environment on growth, efficiency of feed utilization, carcass fatness, and their association. Poultry Science, 74(2), 285-296. https://doi.org/10.3382/ps.0740285

Sullivan, M. S. (1991). Flock structure in red junglefowl. Applied Animal Behaviour Science, 30(3-4), 381-386. https://doi.org/10.1016/0168-1591(91)90143-L

Svensson, E., Råberg, L., Koch, C., & Hasselquist, D. (1998). Energetic stress, immunosuppression and the costs of an antibody response. Functional Ecology, 12(6), 912-919. https://doi.org/10.1046/j.1365-2435.1998.00271.x

Szamado, S. (2008). How threat displays work: species-specific fighting techniques, weaponry and proximity risk. Animal Behaviour, 76(5), 1455-1463. https://doi.org/10.1016/j.anbehav.2008.07.010

T

Temple, W., Foster, T. M., & O'Donnell, C. S. (1984). Behavioural estimates of auditory thresholds in hens. British Poultry Science, 25(4), 487-493. https://doi.org/10.1080/00071668408454890

Tibbetts, E. A., & Izzo, A. (2010). Social punishment of dishonest signalers caused by mismatch between signal and behaviour. Current Biology, 20(18), 1637-1640. https://doi.org/10.1016/j.cub.2010.07.042

U

V

Veenit, V., Cordero, M. I., Tzanoulinou, S., & Sandi, C. (2013). Increased corticosterone in peripubertal rats leads to long-lasting alterations in social exploration and aggression. Frontiers in behavioral neuroscience, 7, 26. https://doi.org/10.3389/fnbeh.2013.00026

Ventura, B. A., Siewerdt, F., & Estevez, I. (2010). Effects of barrier perches and density on broiler leg health, fear, and performance. Poultry Science, 89(8), 1574-1583. https://doi.org/10.3382/ps.2009-00576

Ventura, B. A., Siewerdt, F., & Estevez, I. (2012). Access to barrier perches improves behaviour repertoire in broilers. PloS one, 7(1). https://doi.org/10.1371/journal.pone.0029826

Versace, E., & Vallortigara, G. (2015). Origins of knowledge: Insights from precocial species. Frontiers in Behavioral Neuroscience, 9, 338. https://doi.org/10.3389/fnbeh.2015.00338

Vestergaard, K. S., Kruijt, J. P., & Hogan, J. A. (1993). Feather pecking and chronic fear in groups of red junglefowl: their relations to dustbathing, rearing environment and social status. Animal behaviour, 45(6), 1127-1140. https://doi.org/10.1006/anbe.1993.1137

Vinkler, M., Bainová, H., & Albrecht, T. (2010). Functional analysis of the skin-swelling response to phytohaemagglutinin. Functional Ecology, 24(5), 1081-1086. https://doi.org/10.1111/j.1365-2435.2010.01711.x

W

Warriss, P. D. (2000). Post-mortem changes in muscle and its conversion into meat. Meat science: An introductory text, 93-105. https://doi.org/10.1079/9780851994246.0093

West, B., & Zhou, B. X. (1988). Did chickens go north? New evidence for domestication. Journal of archaeological science, 15(5), 515-533. https://doi.org/10.1016/0305-4403(88)90080-5

Wichman, A., Heikkilä, M., Valros, A., Forkman, B., & Keeling, L. J. (2007). Perching behaviour in chickens and its relation to spatial ability. Applied Animal Behaviour Science, 105(1-3), 165-179. https://doi.org/10.1016/j.applanim.2006.05.009

Widowski, T. (2010). The physical environment and its effect on welfare. In The Welfare of Domestic Fowl and Other Captive Birds (pp. 137-164). Springer, Dordrecht.

Widowski, T., & Torrey, S. (2018). Rearing young birds for adaptability. In Advances in Poultry Welfare (pp. 49-76). Woodhead Publishing. https://doi.org/10.1016/B978-0-08-100915-4.00003-8

Wood-Gush, D. G. M. (1959). A history of the domestic chicken from antiquity to the 19th century. Poultry Science, 38(2), 321-326. https://doi.org/10.3382/ps.0380321

Wood-Gush, D. G. M., & Rowland, C. G. (1973). Allopreening in the domestic fowl. Revue Comportement Animal, 7, 83-91.

Wood-Gush, D. G. M. (1971). The behaviour of the domestic fowl (No. 7). Heinemann Educational Books Ltd..

X

Y

Yahav, S., Straschnow, A., Plavnik, I., & Hurwitz, S. (1996). Effects of diurnally cycling versus constant temperatures on chicken growth and food intake. British Poultry Science, 37(1), 43-54. https://doi.org/10.1080/00071669608417835

Yngvesson, J., & Keeling, L. J. (2001). Body size and fluctuating asymmetry in relation to cannibalistic behaviour in laying hens. Animal behaviour, 61(3), 609-615. https://doi.org/10.1006/anbe.2000.1616

Yunianto, V. D., Hayashit, K., Kaiwda, S., Ohtsuka, A., & Tomita, Y. (1997). Effect of environmental temperature on muscle protein turnover and heat production in tube-fed broiler chickens. British Journal of Nutrition, 77(6), 897-909. https://doi.org/10.1079/BJN19970088

Z

Zajonc, R. B., Wilson, W. R., & Rajecki, D. W. (1975). Affiliation and social discrimination produced by brief exposure in day-old domestic chicks. Animal Behaviour, 23, 131-138. https://doi.org/10.1016/0003-3472(75)90059-7

Zidar, J., Campderrich, I., Jansson, E., Wichman, A., Winberg, S., Keeling, L., & Løvlie, H. (2018). Environmental complexity buffers against stress-induced negative judgement bias in female chickens. Scientific reports, 8(1), 1-14. https://doi.org/10.1038/s41598-018-23545-6

Zimmerman, P. H., Lindberg, A. C., Pope, S. J., Glen, E., Bolhuis, J. E., & Nicol, C. J. (2006). The effect of stocking density, flock size and modified management on laying hen behaviour and welfare in a non-cage system. Applied Animal Behaviour Science, 101(1-2), 111-124. https://doi.org/10.1016/j.applanim.2006.01.005

Zuk, M., Thornhill, R., Ligon, J. D., Johnson, K., Austad, S., Ligon, S. H., ... & Costin, C. (1990). The role of male ornaments and courtship behaviour in female mate choice of red jungle fowl. The American Naturalist, 136(4), 459-473.

ACKNOWLEDGMENTS/

AGRADECIMIENTOS

Acknowledgements/ Agradecimientos

Primero de todo quería dar las gracias a mi directora de tesis Inma Estévez por haberme dado esta gran oportunidad y por ser mucho más que una directora. Gracias por transmitirme tu gran pasión por la etología y el bienestar animal, tus ganas de conocer y saber más. Por incluirme en tu equipo y enseñarme tanto… Gracias por apoyarme, por tu paciencia y por tus ánimos. Eres una luchadora y una gran profesional. Sabes que siempre te llevaré en mi corazón.

Gracias a todo el departamento de producción animal de Neiker-Tecnalia. A Roberto, Eva, Ina, Josune, Aser, Larraitz, Lourdes, Hanen, Carolina y a todos los estudiantes en prácticas o de master que habéis pasado por aquí y que tanto nos habéis ayudado. Y gracias a las chicas de administración, al equipo de informática y por supuesto al de mantenimiento que tanto nos ayudó en la construcción de los recintos y demás abalorios para las gallinas. Gracias a todos por hacerme los días más alegres.

Especialmente, gracias a los que compartimos torreta y horas codo con codo. A Xavi por nuestras interminables charlas, cafés, cervezas y por su inestimable ayuda. Por su cariño, las risas y por todo su apoyo cuando lo necesitaba. Gracias también por haber compartido los largos viajes de vuelta a casa. Muchísimas gracias a mis queridas Ane y Joanna, por haber sido compañeras de vida y de trabajo, de pasión y diversión. Sin vosotras esta etapa no hubiera sido lo mismo. Gracias Joana por las risas y los dramas. Por compartir conmigo tu mundo. Gracias Ane por animarme a tirar esa beca para llegar hasta aquí. Sabes

que sin ti no hubiera sido lo mismo. Por tu brillo de ojos y tu energía. Por seguir estando a mi lado. Os quiero mucho.

Gracias a Guiomar por tu supervisión y ayuda en el Proyecto, gracias a ti y a Iurancha por tantas jornadas divertidas, por vuestro apoyo y por compartir las largas horas de observaciones en la granja, tiñendo el pelo de las gallinas, etiquetandolas, construyendo recintos o sacando huevos del gallinero.

Muchas gracias a Linda Keeling por haberme acogido y cuidado tanto cuando estaba en Suecia. Gracias por todo lo que me has enseñado. Eres una gran mujer, una gran científica y una gran profesora. Muchas gracias también a todo el equipo de la SLU que tanto me enseño y ayudo. Especialmente a Sonja, Rebecca, Dianna, Anette, Elena, Josephina, Ana y Erika por su apoyo y gran ayuda en el experimento que allí realizamos. Gracias por ser un gran equipo.

Gracias a Nico Názar por compartir parte de mis experimentos, por pasar largas horas con nosotras y por corregir mi tesis y los artículos. Por todas las risas y por todo tu apoyo. Gracias también a Raúl Marin por formar parte de este proyecto. Por ayudarnos tanto con tus ideas y experiencia. Un abrazo fuerte para Argentina.

Gracias a David Lizaso y Esteban Atxa por su ayuda con la gestión de la granja, los huevos, las vacunas y las gallinas.

Gracias a mis queridas gallinas por permitirme conoceros un poco más.

Gràcies a en Josep Maria Farreny per ajudar-me a trobar el meu pròpi camí quan em sentia perduda.

Gràcies també als meus pares, als meus germans y a les seves famílies. Gràcies als meus sogres, cunyades i cunyats. Gràcies a tots per estar sempre al meu costat, per estimar-me tan, per la vostra paciència y ajuda. Us estimo molt.

Gràcies a en Manel, per ser el meu company de viatge i de vida, per animar-me sempre que ho he necessitat. Per estar al meu costat des de l'inici d'aquesta aventura i per mantenir-t'hi. Per tenir tanta paciència i per donar-me tan amor. Gràcies per confiar en mi i ajudar-me a créixer. Gràcies també a la nostra filla Emma, per ser la nostra alegria, la que ens omple de llum cada dia. Ets emoció i alegria en estat pur. I gràcies a la filleta que està per vindre i que ens morim de ganes de conèixer. Vosaltres ens doneu força per reinventar-nos i per intentar ser cada dia una mica millors.

Aquest doctorat representa moltes coses per mi, penes, alegries, frustracions i somnis, ha set una part molt important de la meva vida i m'ha marcat en molts sentits. Gràcies a la vida per aquesta experiència sense la qual jo no seria qui soc. Gràcies a tots aquests éssers estimats que heu estat d'una manera o una altre al meu costat durant el desenvolupament d'aquest projecte, tan si us nombro com si no, us estimo, sense vosaltres no ho hauria aconseguit.