Start on the Right Foot: Choice in a Complex Early Environment Boosts Coping Abilities in the Domestic Fowl Chick

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Abstract

Early experience of a complex environment can improve biologically relevant traits related to coping abilities. However, the mechanisms underlying these positive effects are not well explored. We hypothesised that the possibility to express a choice, leading to increased control over the environment, could play an important role. In a balanced design, laying hen hatchlings were reared either in a 'No-Choice' environment (single litter and perch type) or a 'Choice' environment (four different litter and perch types). Immunological and behavioural indicators of birds' coping abilities were explored at three weeks of age. Chicks from Choice environments had higher natural antibody titres, lower H/L ratios, required more attempts for tonic immobility induction and were more successful gaining novel food rewards in a repeated challenge test. Results imply that their improved natural immune response better prepared Choice chicks for coping with pathogenic challenges, that they experienced less chronic stress and were less fearful. To conclude, the increased potential for control and stimulation arising from making decisions in an early complex environment seemed to better prepare birds for a variety of challenges to come, boosting their adaptive capacities and their ability to make the most of opportunities.

Introduction

Early life experiences are of pivotal importance for the ontogeny of animals [1–4]. In avian species, hatchlings encounter an entirely new environment, far from the security offered by the *in-ovo* situation. This new environment is full of challenges, ranging from getting rid of the shell to exploring this new world and resisting a series of potential pathogens [5–7]. The environment that individuals encounter in the early stages of their development and their genetic potential to respond will, according to the Adaptive Development Plasticity theory [8], determine their later phenotype and thereby affect their fitness [8–10]. With this framework, the particular way each individual responds to challenges can be understood as a reflection of its current abilities but, at the same time, it can also be an early indicator of its future potential.

Poultry species are the result of centuries of selective pressure, aimed at improving certain traits of interest (particularly productive ones) with the purpose of providing humans with derived products [11]. There is considerable evidence that these selective processes have been detrimental for key biological traits related to the ability to cope with stressors or to adapt to new or variable environments [12–16]. Such findings emphasize the need for action to expand and potentiate the adaptive capacities in the domestic strains and one such way is by improving how we rear them.

The rearing environments for modern poultry species are considerably more barren than the complex forest habitat of their ancestor [17–20]. Environments that allow a greater expression of natural behaviour or provide higher environmental complexity during rearing are proposed as beneficial for success later, in the adult environment [18]. Simple rearing environments, such as floor systems or cages, are likely not cognitively stimulating or spatially complex enough to prepare pullets to navigate in an aviary or free

range laying systems [21,22]. Highly relevant developmental processes occur in the first weeks of life, and this early temporal window could influence coping-related abilities [23–25]. A poor environment during early life may incur costs simply because of the lack of stimulation necessary for optimal development [4,26,27]. That a specific early input could result in a phenotype of general better fitness is also in line with the somatic state-based type of adaptive developmental plasticity [8].

Early experiences in complex environments have been reported to have direct and long-lasting effects on a series of biologically relevant variables related to the interaction of birds with their environment. Zidar et al. (2018) demonstrated that additive stress can have a negative influence on behaviour and cognitive processes in domestic chicks, and that environmental complexity by providing perches and shelter, can buffer against these negative effects. In another experiment with laying hens, the presence of cage furnishings from 3 weeks of age positively influenced physiological indicators and resulted in hens from furnished cages showing higher innate immune responses and antibody production, indicating improved adaptability, compared to stressed cage-reared and the control (non-stressed, no furnishings) cage-reared hens [29]. Additionally, Campderrich *et al.* (2019) showed that provision of a complex environment (with perches and shelter) helped reduce both the negative effects of a previous exposure to cold stress, as well as enabling birds to better handle a variety of stressors encountered later in life. Thus early experience had both immediate and long-lasting effects on the modulation of their coping-phenotypes [30].

Most studies investigating the effect of early environment on poultry welfare, behaviour and physiology focus on the comparison of a barren versus an enriched early environment. Negative consequences of the specific characteristics of a barren environment, such as limited access to ground substrate [31–34] or limited acces to perches [22,35], have been reported, among other things, to result in increased mortality and abnormal behaviour, including severe feather pecking and cannibalism later in life. It is nevertheless interesting to deepen our understanding of how early environmental complexity can alter birds' abilities to cope with different challenges. Environmental complexity can be increased by offering different resources (the usual approach), but it can also be increased by offering different forms of the same resource. Variation in the environment, increases individuals' possibilities to choose, which in turn leads to increased control, something that has been stated as crucial for the well-being of an individual [36]. It is possible that it is the experience of making choices that underlies the positive effects found for early complex environments.

The aim of this study was to elucidate the effects of early environmental complexity differing in levels of choice on biologically relevant traits related to coping abilities in 3-week-old chicks. We designed two early environments which differed in complexity by providing either a choice (Choice environment) or no choice (No-Choice environment) of different types of substrate and perches, two resources already known as important for laying hen chicks [37]. Variables representative of fear and stress responses as well as variables to explore the immune status were selected. By providing substrate and perches in both treatments, our No-Choice environment corresponded to the 'complex environments' that have so far been used in previous studies [28,30]. Our Choice environment then implies a further increase in the complexity

but without the addition of further resources. We hypothesise that this increased complexity will better boost behavioural and physiological coping abilities in the chicks reared in the Choice environment. In practice this would mean they were better prepared for the challenges involved in commercial poultry production systems.

Methods

Animals and husbandry

Day-old laying hen chicks of the hybrid Bovans Robust were purchased from a Swedish commercial hatchery (Swedfarm AB). After the standard procedure of handling and sorting at the hatchery, they were transported 255 km in a temperature-controlled compartment to the Swedish Livestock Research Centre. On arrival, all 104 birds were weighed and placed in one of eight pens in an indoor facility. The birds were randomly assigned to each pen, but checked so there were no significant differences between pens in the average weight per bird (average weight per bird was 36.2 ± 0.13 g). Each pen was 1.2 x 2.4 m and housed 13 birds. The birds were provided with water and feed (commercial standard) *ad libitum* throughout the whole study. The light intensity was on average 20.2 ± 1.67 lux in the pens. The birds had heat lamps hanging above the middle of the pen during the whole experiment. The average temperature under the heat lamps was 43.6 ± 1.65°C and 23.6 ± 0.26°C in the rest of the pen. All birds were marked with numbered leg rings at two weeks of age. The experiment was conducted between 13th of May and 4th of June 2019. At the end of the experiment, when birds were 22 days old, they were weighed.

Environmental treatments

Pens were assigned before chick arrival to one of two environmental treatments; “No-Choice” (only one substrate type and one perch design on all four locations, Fig. 1a) or “Choice” (four different ground substrates and four different perch designs in the pen in different locations, Fig. 1b). The substrates were straw, wood shavings, sand and peat. The perch designs were round rubber, braided rope, flat wood and flat wire. The substrates and perch types were chosen due to their different characteristics, as well as based on a pilot study showing that different substrates and perches were preferred for different types of behaviour [38]. Test substrates were presented in four trays (71 x 35 x 3.5 cm, see Fig. 1). A low stocking density was used so that all chicks could potentially use a particular litter box or perch at the same time. Each of the substrate types and perch designs presented in the Choice environments were presented in one No-Choice environment (Fig. 1). A substrate outside the trays was used to compensate for the coldness of the concrete floor for the young chicks. It was selected so that each substrate type was present in one Choice treatment pen and they matched the test substrates in the No-Choice treatment pens. Perch height started at 15 cm and was elevated at 14 days to 45 cm. The locations of the different treatments were balanced across the room. Pen walls were covered to minimize visibility between neighbouring pens.

Immunological variables

Based on previous studies [30,39,40], four variables were investigated to assess the status of each individual's immune system; 1) The lymphoproliferative response to phytohaemagglutinin-p (PHA-P), a cellular representative of the immune system reflecting birds' pro-inflammatory potential [41], 2) Interferon gamma (IFN-) plasmatic concentration, as a pro-inflammatory mediator 3) Natural Antibodies against sheep red blood cells (SRBC), reflecting general humoral immune capacity, and 4) Heterophil/Lymphocyte (H/L) ratio, a representative of the cellular immune response as well as an haematological indicator of underlying chronic stress responses.

The sampling procedure lasted two days and started when birds were 16 days old. The lymphoproliferative response required an intradermal injection and an *in-vivo* analysis the day after, while the other three variables were analysed *in-vitro* with blood sampled on one occasion. Blood was sampled 24 hours post lymphoproliferative induction.

The lymphoproliferative or *swelling response to PHA-P*. The amount of 0.05 ml solution of PHA-P (Phaseolus vulgaris lectin from Sigma Aldrich) in PBS (1 mg / 1 ml solution) was injected in the left wing web of each bird according to previous descriptions [30,41,42]. After 24 hours (± 1.5 hours), the thickness of the pre-marked injection site was measured and compared with basal thickness, measured just before the injection. The thickness was measured using a digital calliper (Cocraft®) with an accuracy of 0.03 mm. The indicator of swelling was obtained using the calculation; Percentage of swelling = (basal thickness / thickness post 24 hours) x 100 [39].

Blood sampling. A maximum of 0.75 ml was obtained from the right brachial vein of each bird (opposite wing from the PHA-P response induction). Syringes were prepared with 0.1 ml of the anticoagulant Ethylenediaminetetraacetic acid (EDTA). Blood smears were made immediately, using one drop from the syringe according to practice, while the remaining blood was placed on ice in a transport box. Blood was then centrifuged at 2000 g for 10 minutes to obtain plasma that was stored at -20°C until further analyses. The same person collected all blood samples.

IFN- plasmatic concentration. IFN- was quantified using a validated species-specific ELISA kit (Ray Bio® Chicken IFN-gamma ELISA Kit, ELG-IFNg). The minimum detectable dose was assessed to be 0.06 ng/mL. Intra- and inter-assay reproducibilities were 10 % and 12 % respectively. Procedures specified by the manufacturer were followed and the concentrations for all birds were determined the same day.

Natural antibody response against SRBC. Natural antibodies (Nab) for SRBC were assessed using a microagglutination assay [43]. Procedures were similar to those conducted for investigating acquired antibody responses for SRBC. An amount of 25 µl complement-inactivated (through a thermal bath at 56°C) plasma was serially diluted in 25 µl of phosphate buffer saline (PBS) (1:2,1:4,1:8 up to 1:512). Then, 50 µl of a 2% suspension of SRBC in PBS was added to the wells. Microplates were then covered with aluminium foil and incubated at 40°C for 1 hour and checked for agglutination every 15 minutes. Haemagglutination of the test plasma samples were compared to the blanks (PBS only) and negative

controls (wells with no SRBC suspension). Antibody titres were reported as the Log^2 of the highest dilution yielding significant agglutination.

H/L ratio. Blood smears were stained with May Grünwald Giemsa and differential counts of 100 white cells per smear were made according to previous practice [30,40]. The heterophil/lymphocyte ratio was then calculated by dividing the number of heterophils by the number of lymphocytes [42,44].

Behavioural variables

The environmental treatment effects on behaviour were evaluated by two behavioural tests, one on an individual level; tonic immobility (TI) test, and one on a pen-group level; repeated challenge test.

Tonic immobility test. The duration of the TI response is thought to reflect an individual's fear level [45] and is frequently used in poultry research. The procedures described by Zidar et al. (2017) were followed. TI was induced by placing a bird on its back and a hand placed on its chest with a light pressure for 15 seconds. If the individual moved within three seconds, induction was repeated a maximum of three times. Individuals who were not induced after three attempts were given a TI duration of one second, otherwise latency to first head movement, latency to first vocalisation and latency to stand up from the tonic position (TI duration) were registered. Individuals still in a tonic position after five minutes were carefully helped to stand up and received the maximum score of 300 seconds. Three different test persons conducted the test according to standardised procedures so that all birds could be tested on the same day within a period of five hours. Treatments were balanced between different experimenters.

Repeated challenge test. The test was constructed to explore birds' abilities to adapt to routine procedures, i.e., repeated exposure to an initially novel situation. The test consisted of two phases with an increased challenge level in the second phase. Each phase consisted of three repetitions and all were carried out in each pen, over a two-day period, when birds were three weeks of age.

In the first phase of the test (repetitions 1–3), a test person opened the entrance door to the pen and placed an initially novel object (a porcelain bowl) with initially novel feed (ten live meal worms) mixed with an initially novel substrate (crushed straw pellets) in the home pen for 90 seconds. The bowl was placed in the middle of the pen, one arm's length from the entrance, before closing the door. Time and video recording started once the door was closed. In the second phase (repetitions 4–6), the challenge level was increased by the test person actually entering the pen and sitting down in the corner by the entrance. She presented two feed bowls (same bowls and content as in the first 3 repetitions) during 90 seconds (see Fig. 2). One bowl was placed on the ground in front of her, in a similar position to in phase 1, whereas the other bowl was held on her lap. She had her gaze downwards and to the right, avoiding eye contact with the birds. An assistant closed the pen door, while the test person sat down. Time and video recording started once the door was closed. Repetitions 1–5 occurred during the first day, with at least one hour between each repetition, while the 6th occurred the day after.

This test can be considered as a series of challenges that the birds have repeated opportunities to overcome in order to access the food reward. Given that the birds were allowed to move initially to the far end of the pen, the latencies to overcome consecutive challenges can be placed in following order; latency to approach the mid and then the near part of the pen (Fig. 2), latency to peck in the bowl on the ground (repetitions 1–6), latency to jump up onto the person and, finally, latency to peck in the bowl on the person's lap (repetitions 4–6). The latency recorded was the time for the first bird in the pen to overcome the challenge. Additionally, from the video recording, the number of birds in each area of the pen (far, mid, near) every 10 seconds and the total number of pecks to each bowl were determined. The total number of worms eaten in each bowl was registered by counting the number of worms remaining in the bowl at the end of the test as an indicator of overall sucess.

All methods were carried out in accordance with relevant guidelines and regulations. The study was approved by the National Ethics Committee for Animal Experiments in Uppsala (5.8.18–11549/2017) and complied with the ARRIVE guidelines [46].

Statistical analysis

Statistical analyses were conducted in R software (version 3.3.2; Development Core Team, 2016).

Linear mixed models, fit by Restricted Maximum Likelihood (REML) and lme4-package [47] were used for variables showing normal distribution and homoscedasticity. Significant fixed effects were investigated using Type III ANOVA with Kenward-Roger's approximation of degrees of freedom and the lmerTest package [48].

For all mixed models, pen was used as a random effect, while treatment was used as a fixed effect. These models were fitted for "swelling response to PHA-P", "weight increase" and "latency to first head movement (TI)". If the random effect was too small, resulting in convergence issues, this was dropped, and a simple linear model was fitted. This was done for "H/L ratio" and "latency to stand up (TI)". For variables showing non-normality and/or heteroscedasticity, average values per pen were explored for treatment effects using Kruskal-Wallis tests and was executed for "natural antibody titres", "IFN-γ plasmatic concentrations" and "number of attempts (TI)".

In the linear mixed models investigating all the variables from the repeated challenge test, interactions between "treatment", "phase" and "repetition" as fixed effects were included. Since the aim of this test was to explore the birds' abilities to improve with each repetition through learning or adaptation, pairwise comparisons of the interaction "treatment", "phase" and "repetition" were always explored if the effect of "repetition" and "phase" or their interaction came out as $p \leq 0.1$ in the anova.

The Tukey method was used to adjust p-values and control for multiple comparisons. Means calculated using mixed models are presented as estimated marginal means and error values show standard errors of the mean. Transformed values are presented as back-transformed, apart from the natural antibody titres.

For integrating information from all the variables with individual bird data, a multivariate approach was used. Linear discriminant analyses using different treatments as *a-priori* categories were used. The different rearing environments “Choice” vs. “No-Choice” were considered as the different classes in this analysis. In this way, within-class distance was minimized and the between-class distance was simultaneously maximized to achieve maximum class discrimination. The used variables (standardized before analysis) were: “swelling response to PHA-P”, “natural antibody titres against SRBC”, “H/L ratio”, “IFN- plasmatic concentrations”, “latency to stand up” and “number of attempts to induce the TI state” in the TI test. A dispersion graph (biplot) was constructed to visualize both the experimental individuals and the variables in the same space.

Results

One bird from a Choice treatment was euthanized during the first week because of a leg injury. Furthermore, blood withdrawal was not successful for all birds, resulting in a lower number of individuals used in the immunology-related analyses where exact numbers are given for each analysis. There was no difference in overall weight gain of the birds between treatments (Choice: 182 ± 3.85 g vs No-Choice: 184 ± 3.85 g; $F_{1,6}$=0.12, p = 0.73).

Immunological treatment effects

A main effect of the environmental treatment was found for natural antibodies against sheep red blood cells (SRBC), where birds reared in a Choice environment had higher natural antibody titres than their counterparts reared in No-Choice environments (x^2 = 5.33, df = 1, p = 0.02; Fig. 3a). A treatment effect was also found for H/L ratios, where birds reared in Choice environments had lower H/L ratios in comparison to birds from No-Choice environments ($F_{1,74}$=6.92, p = 0.01; Fig. 3b). No effect of the environmental rearing condition was found on the inflammatory response to PHA-P (Choice = 94.2 ± 3.03; No-Choice = 95.3 ± 3.03; $F_{1,6}$=0.06, p = 0.81) nor on the IFN- plasmatic concentration (Choice = 10.96 ± 1.84; No-Choice = 11.13 ± 2.18; x^2 = 0.08, df = 1, p = 0.77).

Tonic immobility test

Compared with birds from No-Choice environments, birds from Choice environments required more attempts to induce the TI state (Choice = 1.14 ± 0.03; No-Choice = 1.02 ± 0.02; x^2 = 4.57, df = 1, p = 0.03) and had a shorter latency to stand up after TI had been induced (Choice = 68.1 ± 10.6; No-Choice = 109.5 ± 17.0; F = 4.67, df = 1, p = 0.03). No treatment differences were found regarding latency to first head movement (Choice = 37.35 ± 9.06; No-Choice = 37.78 ± 9.16; $F_{1,6}$=0.001, p = 0.97) or first vocalization (Choice = 39.14 ± 3.86; No-Choice = 37.39 ± 10.20; x^2 = 0.19, df = 1, p = 0.66).

Repeated challenge test

During the first phase of the repeated challenge test, birds from both environments showed significant reductions from the first to the third repetition, in their latency to reach the mid (Choice: t = 2.45, df = 30, p

= 0.05) or near area of the pen (Choice: t = 2.73, df = 30, p = 0.03; No-Choice: t = 3.96, df = 30, p = 0.001) and in their latencies to start pecking in the ground bowl (Choice: t = 4.83, df = 30, p ≤ 0.001; No-Choice: t = 4.22, df = 30, p ≤ 0.001; Fig. 4). In the second phase, only birds from the Choice environment showed improvements in the consecutive challenges shown in Fig. 4. Significant reductions in latencies in this phase, were found for latency to reach near area (Choice: t = 3.96, df = 30, p = 0.001), to start pecking in ground bowl area (Choice: t = 2.91, df = 30, p = 0.02), to jump up on the person (Choice: t = 2.76, df = 30, p = 0.04) and to start pecking the top bowl (Choice: t = 2.77, df = 30, p = 0.04).

Compared to the first phase, pens in the second phase of the test (when the experimenter was in the pen), had a lower average proportion of birds in the near area (0.55 ± 0.04 vs 0.25 ± 0.04; $F_{1,30}$=100.37, p ≤ 0.001; Fig. 5a), a higher average number of pecks (68.4 ± 6.39 vs 38.2 ± 6.39; $F_{1,30}$=30.2, p ≤ 0.001; Fig. 5b) and a lower average proportion of eaten worms (0.58 ± 0.03 vs 0.82 ± 0.04; $F_{1,27}$=29.62, p ≤ 0.001; Fig. 5c).

In pairwise comparisons investigating how birds from each treatment improved with repetition, there was no increase in the proportion of birds in the area near the novel bowl in the first phase (p > 0.05). However, in the second phase, birds from a Choice environment showed a significant increase in the proportion of birds in the near area between the fourth to sixth repetition (t=-2.47, df = 30, p = 0.049). No equivalent increase was found for birds from a No-Choice environment (Fig. 5a). Birds from both environments showed an increase in number of pecks with repetition in both phases (p ≤ 0.05; Fig. 5b) and in the proportion of worms eaten in first phase (p ≤ 0.05; Fig. 5c). However, in the second phase there was only a significant increase in the proportion of eaten worms for Choice environments (t=-2.78, df = 30, p = 0.025; Fig. 5c).

Multivariate environmental treatment effects

Figure 6 shows a lineal discriminant analysis using those variables measured individually in the birds in this study: inflammatory response against PHA-P, Nab production against SRBC, H/L ratio, IFN-γ plasmatic concentrations, weight gain, latency to stand up and and number of attempts to induce the tonic state, in a TI test. The two environmental treatments are defined by the distribution of the coloured dots in the discriminant space determined by the canonical axes. The figure shows an effective discrimination of the individuals according to their *a-priori* treatment: reared in Choice or No-Choice environments. This discrimination can be clearly observed in Canonical axis 1 (94.9% of variability between the groups explained), for which natural antibody titres against SRBC and the heterphil/lymphocyte ratio are the two most important (discriminant coefficients 0.98 and 0.20, respectively).

Discussion

Birds reared in an environment where there was a choice of different substrates and perch types showed improved immune potential, indicators of diminished fear and stress responses, as well as increased exploratory traits compared to birds reared in a similar environment but without choice within these

resource types. These results support our hypothesis that the increased complexity achieved by providing more choices in the environment, altered the phenotype of the chicks by boosting their coping abilities. In practice, this better preparation for environmental challenges could be a practical way to promote improved bird welfare.

The novelty of this work lies in how we changed the complexity of the environment. Enhanced complexity is usually achieved by adding diverse resource types to the environment, whereas we have varied their form, thereby giving birds choices within each resource type of where to perform different behaviours. Additionally, we have analysed immediate effects (those found during the first three weeks of life) whereas most of the previous research in the area of early environmental manipulations in domestic birds falls within one of two categories: 1) prenatal/parental and *in-ovo* manipulations (reviewed in [7]), or 2) early manipulations with effects analysed later in life (youth or adulthood) [18,28,49–51]. Maternal passive immunity protection lasts until about two weeks post hatch [52,53] so our variables were collected when the chick is learning to rely on its own immune system.

We first discuss results from the immune related variables that were selected as indicators of the birds' competence to get rid of potentially pathogenic challenges, using non-pathogenic techniques. We then go on to discuss the behavioural results and how they relate to a bird's ability to learn in new and potentially challenging situations, as well as how success in such a situation may be influenced by fearfulness. Finally, we return to the broader issue of how early environment can influence the later phenotype and how that knowledge might be used to advantage when rearing commercial laying hens.

A difference was found when quantifying natural antibodies where birds reared in choice environments showed higher circulating concentrations. Natural antibodies are present in non-immunized individuals and cover a broad specificity repertoire [54,55]. They originate from continuous stimulation by exogenous microbes, or correspond to the secretion of naturally occurring auto-reactive B cells, or both [56]. It is likely that the environment with choice, especially due to the various substrate types, could have had a more diverse microbe community, greater pathogenic load and a wider pathogenic diversity (as previously proposed for enriched conditions [39,57]) that triggered the higher production of natural antibodies. Natural antibodies are of great importance because they are key to activating other immunological compartments, such as the complement system and adaptive immune responses [54,58,59]. It was also the variable that had greatest discrimation power between our treatments. Our results suggests that chicks reared in an environment with various substrates and perch types, had the advantage of a potentially better prepared immune system compared to those with the same allocation of enrichment resources but no variation. In the long run, survival would be enhanced based on studies that have proposed a relation between elevated natural antibody concentration and the increased probability to survive the laying period in hens [58].

No effect of environmental choice was found on the *in-vivo* pro-inflammatory potential nor in the IFN- concentrations, which implies chicks were equally prepared to deal with potential pathogens requiring

inflammatory milieus for its clearance [40,41]. This information gives a clue, for the first time, about the specificity of the effects of the increased environmental complexity mediated by choice, and points towards enhanced immunological potential related to humoral mediators and the series of responses that depend on natural antibodies being activated. This would provide Choice-chicks with the advantage of a potentially faster activation of these responses, so reducing time and energy allocated to immune coping.

Regarding the behavioural variables, both the tonic immobility and the repeated challenge test were able to identify specific treatment effects that also support our hypothesis that increased environmental choice during the first weeks of life would improve the coping abilities of young chicks. Birds reared in the Choice environments required more attempts to induce the tonic immobility state and showed a shorter latency to stand up, suggesting that they were less fearful than birds from the No-Choice environments [45]. Numerous previous studies have found that increased environmental complexity by adding enrichments, can result in less fearful birds [60–62]. This is advantageous from a welfare perspective, since the production environment usually contains various potential stressors that can lead to fear states and increased fear has been found to be associated with negative consequences such as increased feather damage, low body weight, egg weight, feed intake and even mortality [63].

The repeated challenge test, specifically constructed and designed for testing our hypothesis, seemed to be successful in identifying differences in the abilities of the groups from the different treatments to adapt to challenges with rewarding opportunities. The lower proportion of birds in the area close to the feed bowls in the repetitions involving a human, supports that the second phase of the test was more challenging (as it was intended to be). In the first phase, both pens showed improvements with repetition, shown as reduced latencies to approach and peck at the food bowl and an increased proportion of eaten worms, thereby indicating increasing exploratory behaviour and some level of adaptation. However, in the second and more challenging phase, only birds from Choice environments showed an improvement over repetitions in these same variables and so were better able to take the opportunity to access the additional food reward.

One possible explanation for the difference in the repeated challenge test and the greater success of birds from the Choice environments, could be that they had experience of approaching and using various forms of resources from day one. We have previously shown, using the same litter and perch types, that chicks prefer certain substrate types for certain behaviours and that different perch types affect their abilities to land on them [38]. This suggests that the Choice environment would have given birds a more diverse training of the behaviours involved around perch use, such as jumping and balancing, as well as increased and diversified foraging opportunities. It is therefore possible that the Choice environment led to improved learning abilities. Learning abilities in farm animals has been shown to affect adaptability in a novel environment [64]. Another possible explanation, now focusing on the lack of success to exploit a new food opportunity in the birds reared in the No-Choice environment, could refer to fear and priming. While repeating a test situation can result in decreased reaction times [65], no such improvements are seen

if the repeated stimuli is experienced as too aversive [66]. That birds from the No-Choice environment were more fearful was supported by the previously mentioned results from the tonic immobility test. There is no reason to expect a difference in food motivation as food was always freely available in all pens.

One could suggest that birds' responses in the repeated challenge test would be comparable to their responses during routine procedures in their home pen, for example, when the caretaker entered to check feed and water supplies. That birds from the No-Choice treatment had a higher H/L ratio, a physiological indicator of underlying chronic stress responses, implies that the birds from this treatment were experiencing more chronic stress. This is in keeping with other studies showing that birds from non- or less-enriched environments have increased circulating chronic stress mediators [18].

In the context of the Adaptive Developmental Plasticity theory [8], the increased stimulation available to birds in the Choice environment seems to have had both immediate and potentially long lasting positive effects. All chicks were obtained from the same hatchery and randomly allocated to treatment, thus the prenatal (*in-ovo*) environment for them could be assumed homogenous [7], restricting the interpretation of the results to the effects of the experimental treatments themselves.

For the individuals from the Choice environment, these effects included, as previously detailed, a lower fear response (tonic immobility, [67,68]), a diminished indicator of underlying chronic stress response (H/L ratio, [42,69]) and an increased immune coping potential (natural antibody production, [59,70]). Birds from the Choice environment also had emergent characteristics as a group, as evidenced by the quicker changes in the behavioural variables (latency to approach and to peck in the feed bowl) and greater success in exploiting novel food sources (proportion of eaten worms) in the repeated challenge test. These traits would together characterise an 'adaptive' phenotype in the context of actual breeding conditions, with enhanced coping abilities for a variety of challenges. The traits found among birds from the No-Choice environment differed from those above and would collectively define a 'less adaptive' phenotype. That the two environmental treatments used in this study each configured a phenotype with particular and differential characteristics is shown in the discriminant analysis.

Our results with chicks reared in environments with Choice could represent an early expression of the silver-spoon phenomenon [4]. Choice chicks had the advantage of growing in a stimulating environment with different resources to 'pick and choose' between, allowing for optimal development regarding the coping capacities studied.

Laying hens' chicks are physiologically ready to process stress already at day one [25] and the experience in commercial hatcheries has been shown to be stressful for chicks [71]. The chicks in our experiment were exposed to the typical husbandry procedures, i.e. incubation, handling, post-hatch feed and water deprivation, being subsequently transported and placed in the poultry barn [69,72]. Our results could suggest that early and increased environmental choice could help to alleviate the effects associated with these routinary, but nevertheless challenging events.

In summary, our results support our hypothesis that increased stimulation arising from having choice in a complex environment would potentiate both behavioural and physiological coping abilities of young chicks. The immunological, stress coping and behavioural results obtained were indicative of laying hen chicks being better prepared for immediate, and potentially for future environmental challenges, while at the same time possessing a greater potential to adapt and so make better use of future opportunities.

Declarations

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Author contributions

NN, LS and LK devised the project and contributed to the design and analysis of the study. LS, KMC and NN performed the experiments and collected the data. NN, LS and KMC analyzed the samples. LS and NN prepared the figures and wrote the first version of the paper with input from LK. LK obtained funding. All authors contributed to the interpretation of the data and approved the final version of this manuscript.

Data availability statement

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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Figures

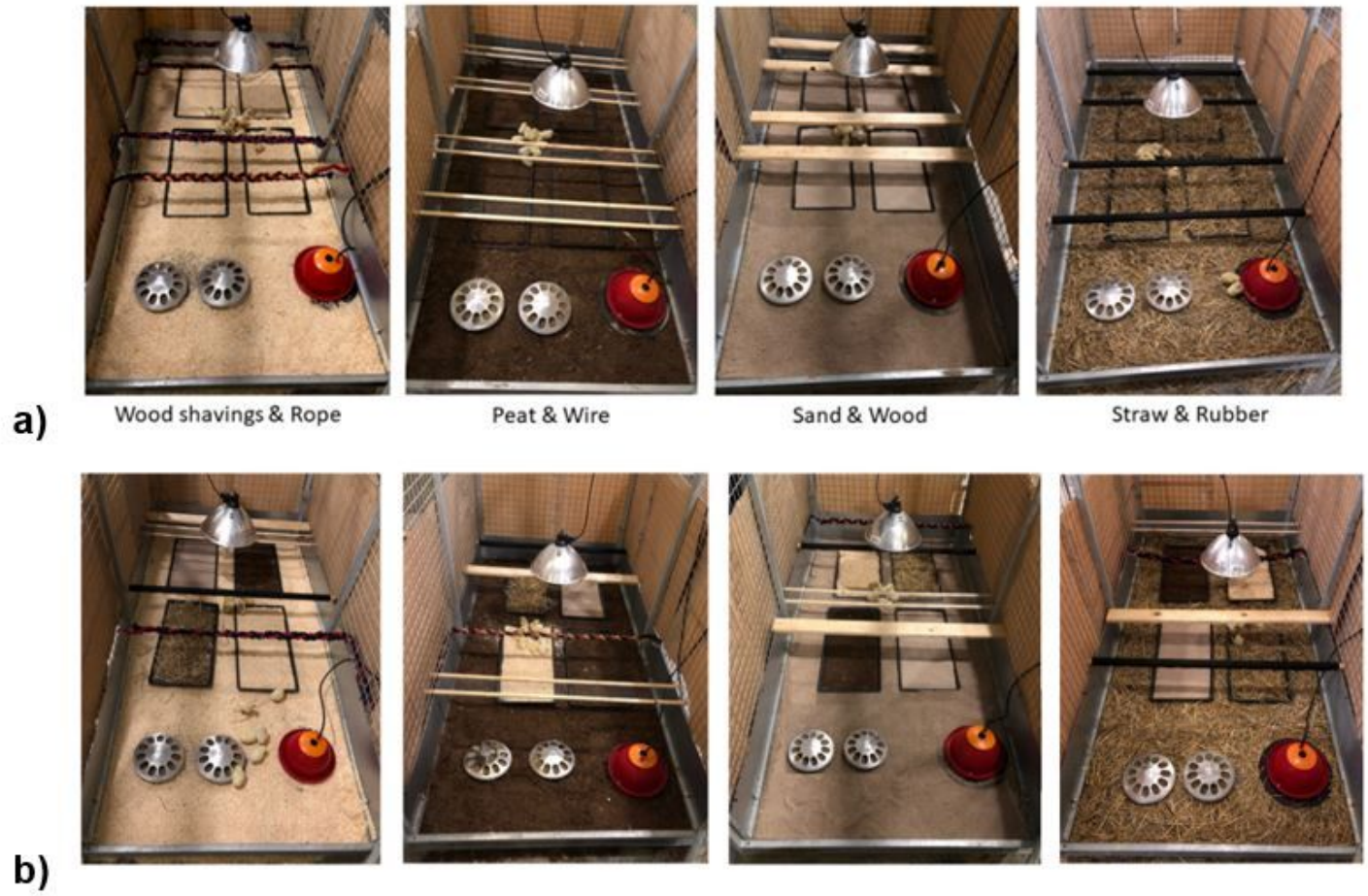

Figure 1

Overview of the environmental treatments. a) the No-Choice environment with only one type of substrate (from left to right: wood shavings, peat, sand and straw) and type of perch in each pen (from left to right: braided rope, flat wire, flat wood and round rubber). b) the Choice environment each with all four types of substrates and perches presented in the No-Choice environments. The location of each substrate and perch type was balanced across the four pens.

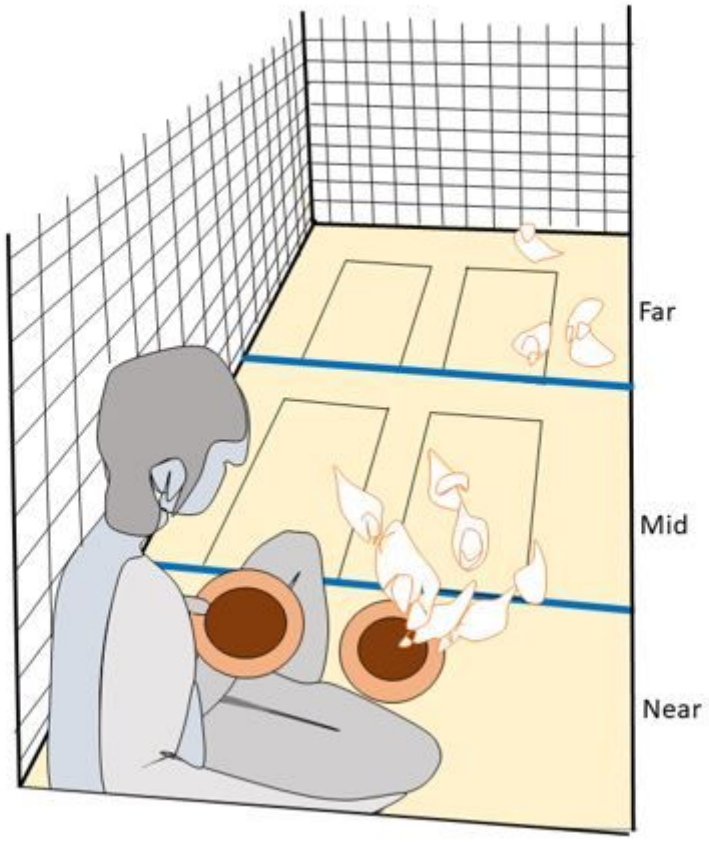

Figure 2

Schematic view of a pen during the repeated challenge test. Each pen was divided in three areas during the repeated challenge test; “far”, “mid” and “near”. During the second phase (the last three repetitions), a test person sat down with legs crossed in the pen placing one feed bowl on the ground and one in her lap as illustrated.

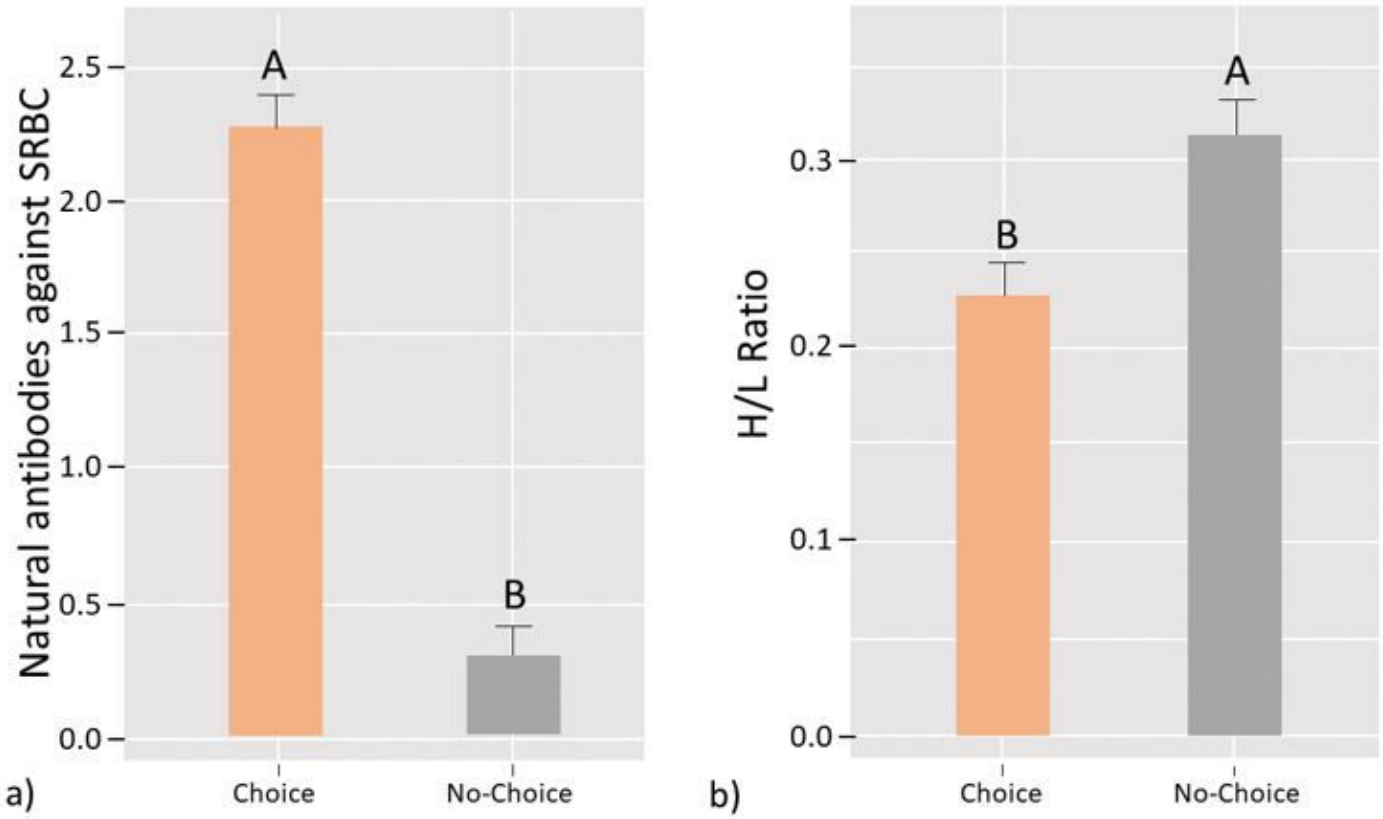

Figure 3

Immunological effects of Choice and No-Choice treatments. Mean and SE of a) natural antibodies against sheep red blood cells (SRBC), presented as the Log2 of the highest dilution yielding significant agglutination (Choice: n=39; No-Choice: n=40) and b) H/L ratio (Choice: n=36; No-Choice: n=39) in blood sampled from 3 week old domestic fowl layer chicks reared in an environmental treatment with Choice or No-Choice. A,B Different letters indicate significant treatment differences.

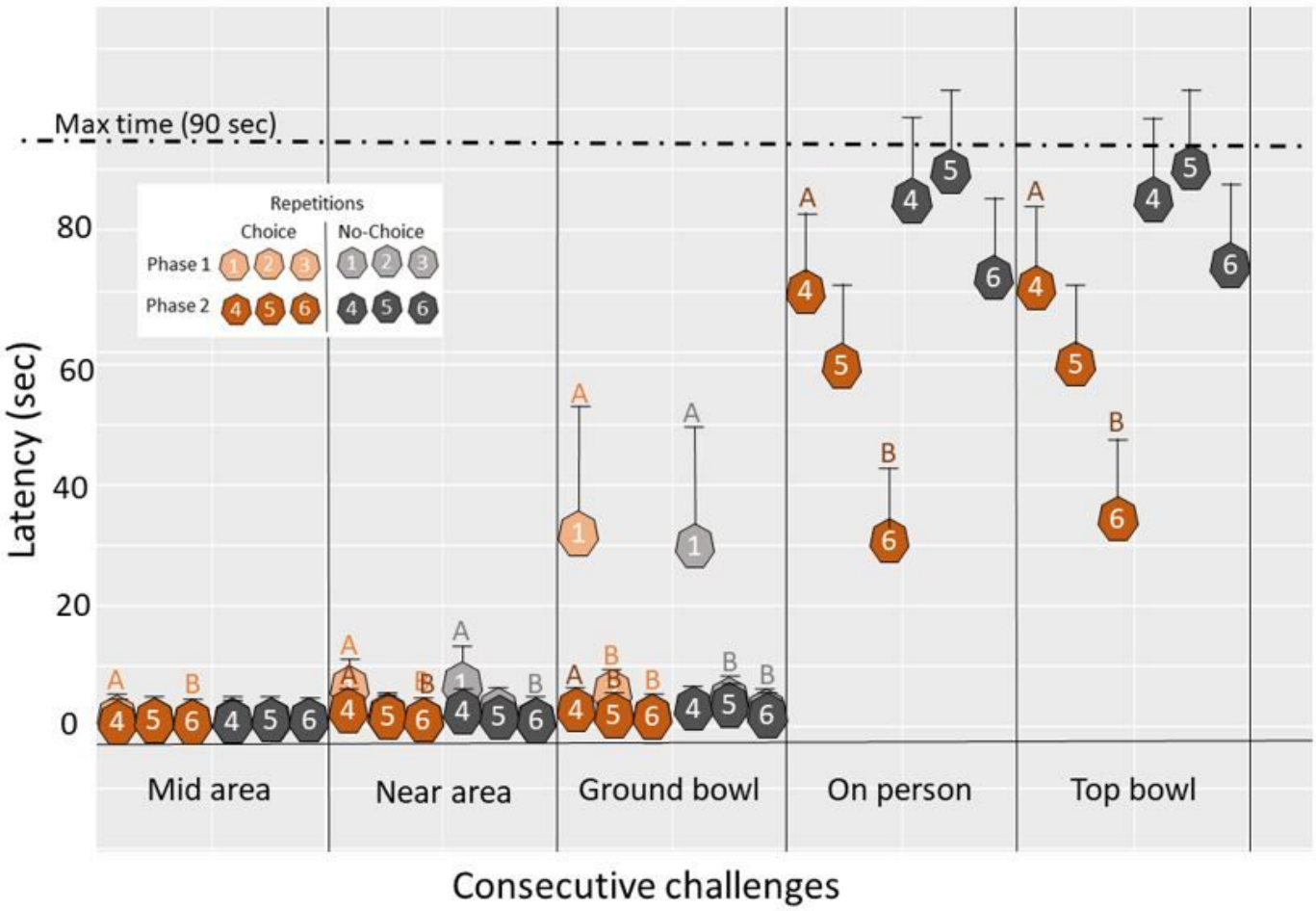

Figure 4

The approach dynamics in the repeated challenge test. Dot plot (Mean and SE) showing the approach dynamics with each repetition during the repeated challenge test of birds from Choice and No-Choice treatments. The first phase (repetitions 1-3) involved one food bowl on the ground, while the second phase (repetitions 4-6) involved a person inside the pen, one food bowl on the ground and another in the lap of the person. Latencies to overcome the challenges are shown in seconds; to enter the mid area, to enter the near area, to peck in the ground food bowl, to jump up on the person where the top food bowl is located and, finally, latency to peck in the top food bowl. A,B Different letters (note also the different shades within each colour) indicate significant differences between repetitions, i.e. improvements with repetition, within each treatment and the different phases of the test. Number of pens per treatment = 4.

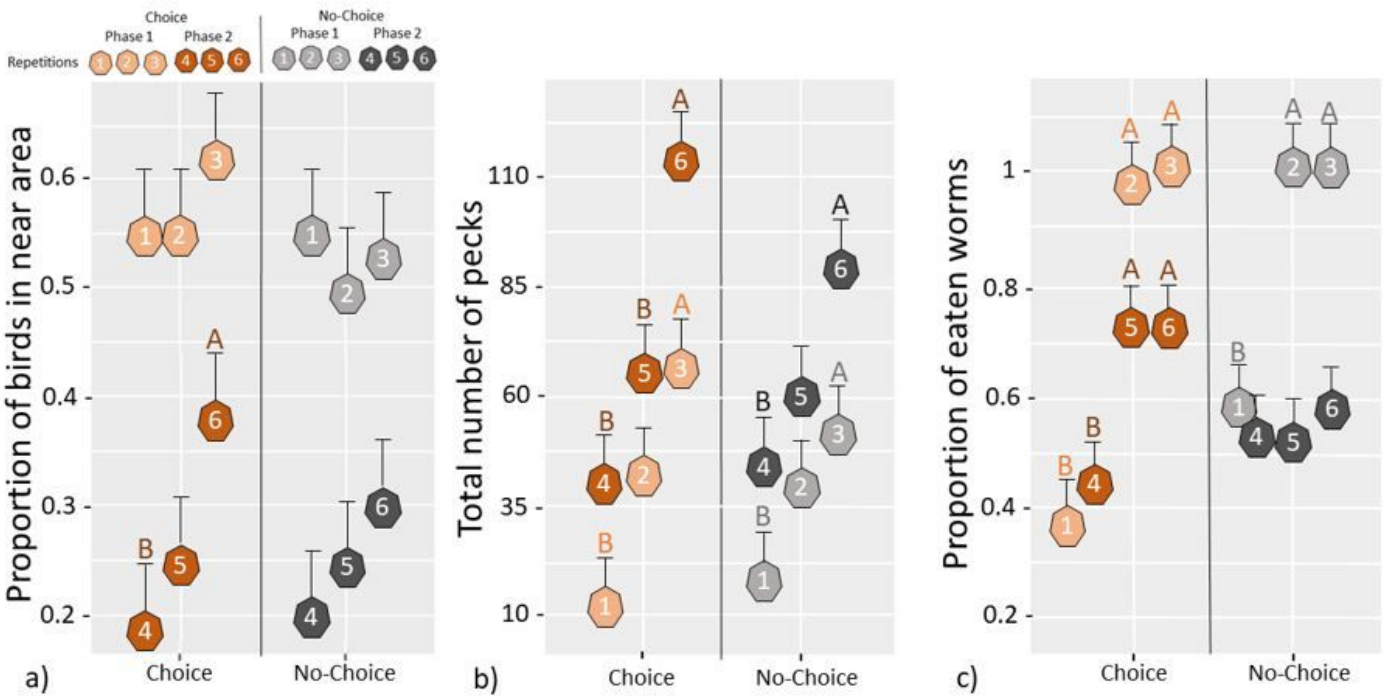

Figure 5

The response dynamics in the repeated challenge test. Dot plot (Mean and SE) showing the response dynamics with each repetition during the repeated challenge test for birds from Choice and No-Choice treatments a) the proportion of birds located in the near area (the area where the ground food bowl was located for repetitions 1-3 and where both food bowls and the test person were located in repetitions 4-6), b) the total number of pecks to feed bowl(s) and c) the proportion of eaten worms during each repetition. A,B Different letters (note also the different shades within each colour) indicate significant differences between repetitions within treatments and phases. Number of pens per treatment = 4.

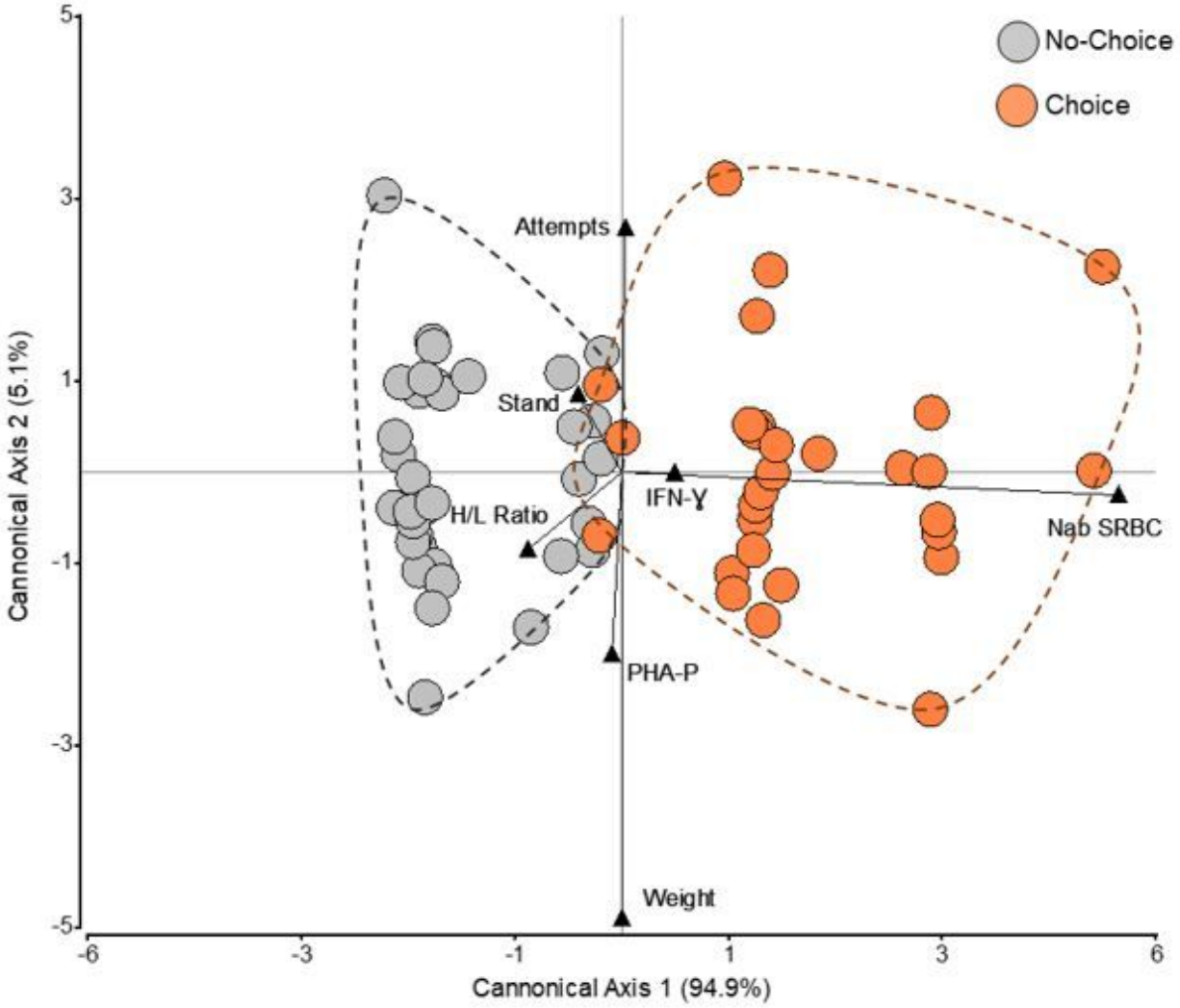

Figure 6

Exploration of the discriminatory capacity of the two environmental treatments. Lineal discriminant analysis including the following standardised variables (shown in black triangles): inflammatory response against phytohaemagglutinin-p (PHA-P), natural antibody titres against sheep red blood cells (Nab SRBC), heterophil/lymphocyte (H/L) ratio, IFN-γ plasmatic concentrations (IFN-γ), latency to stand up in a tonic immobility test (Stand) and number of attempts to induce the TI state (Attempts) and weight gain (Weight). Each dot represents a laying hen chick in the study for which the register of all variables is complete. Grey dots represent chicks reared in the No-Choice environment (n=37), whereas orange dots represent chicks reared in the Choice environment (n=32).