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▼ **EFFECT OF THE n-3 LEVEL ON HEN DIET WITH n-6/n-3 CONSTANT RATIO AND LEARNING ABILITY OF ONE DAY-OLD PARTRIDGE CHICKS (*Alectoris rufa rufa* L.)**

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# ▼ EFFECT OF THE n-3 LEVEL ON HEN DIET WITH n-6/n-3 CONSTANT RATIO AND LEARNING ABILITY OF ONE DAY-OLD PARTRIDGE CHICKS (Alectoris rufa rufa L.)

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## ABSTRACT

In a previous study, we saw that PUFA and particularly n-3 content of partridge parent diet affect learning ability and memory retention of day-old offspring. An n-3 diet content of 4.04 g/kg and an n-6/n-3 ratio of 3.1 produced the best behavioural score. Since references suggest that in mice an n-6/n-3 ratio of 4 affects behaviour more than absolute PUFA and n-3 level, we studied the effect of n-3, with the same n-6/n-3 ratio, on learning ability and memory retention of day-old partridge chicks.

Obtained from eggs laid on the 4$^{th}$ laying week, 144 day-old chicks were tested by mean of a one trial Passive Avoidance Task (PAL task). A negative experience consisting in allowing the chick to peck a coloured (red) bead bathed in a bitter liquid (Methyl Anthranilate) was used. Parents were fed three different diets containing 2.93, 4.06 and 7.60 g/kg of n-3 with an n-6/n-3 ratio of 3.5.

Results showed that when n-6/n-3 ratio of 3.5 is used in laying partridge diet the best behavioural score is expressed by the offspring of the parents fed the highest n-3 content. In our experiment, in fact, the group containing the highest n-3 level showed the best behavioural scores ($p<0.05$); significant lower latency time and higher number of pecks given toward the right (blue) bead.

## INTRODUCTION

The long-chain polyunsaturated fatty acids and particularly arachidonic acid (C20:4 n-6) and docosahexaenoic acid (DHA; C22:6 n-3) are abundantly represented in the brain, retina and other neural tissues (Fenstrom, 1999). DHA constitutes the major n-3 fatty acid in the structural lipids of grey matter and it is essential for optimal brain development and function (Bourre, 2004). During embryo development, the phospholipid membranes of the different tissues acquire a distinct fatty acids composition. The fatty acids profile of the yolk can be modified both by the genetic and by the diet (Surai *et al.*, 2001). In particular, for example, some studies showed that the modification of the linoleic/a-linolenic (18:3 n-3) and the n-6/n-3 fatty acid dietary-ratio, can affect their content in the cells membrane lipids (Ajuyah *et al.*, 2003). Since game birds, can be granivorous, herbivourous, insectivorous or piscivorous, their natural diet can be drastically modified when they are reared in captivity conditions. These diet modifications may affect the brain development, functionality and, therefore, the chick behaviour (Bagliacca *et al.*, 2000; Fronte *et al.*, 2008), as also happens in other species (Wainwright, 2002).

The day-old partridge chick's learning ability whose parents were fed with an n-3 diet level of 4.04 g/kg (in comparison to 0.48 and 7.60 g/kg) and an n-6/n-3 diet-ratio of 3.1 (in comparison to 28.6 and 1.5) showed the best behavioural score in a previous study (Fronte *et al.*, 2008). In addition, Wainwright *et al.* (1999) reported that in mice the best behavioural score showed when the n-6/n-3 ratio was of about 4.

For this reason, the aim of the present study was to detect the effect of the partridge laying hen n-3 diet-content on learning ability of the offspring (day-old chicks), using similar n-3 diet-content but with a constant n-6/n-3 diet-ratio, 3.5 approximately. The use of this n-6/n-3 diet-ratio, was necessary to avoid the use of a very high diet fat content in comparison to the diet commonly used for these birds.

## MATERIALS AND METHODS

Animals and Diets - Three different parent groups, named Low, Medium and High, were fed with three different diets containing 2.93, 4.06, 7.60 g/kg, respectively. All the considered diets were characterized by the same n-6/n-3 ratio of 3.5. Each group, 48 red-legged partridge pairs each, 2 and 3 years old, homogeneously distributed in the groups, were randomly allocated in pair cages kept outdoors (45 x 80 x 35 cm - 1 x 1 cm wire mesh floor). Egg laying was obtained under an artificially extended photoperiod (natural + artificial 16L:8D total; 35 lux artificial light intensity). The eggs laid in the $4^{th}$ laying week were used. The eggs, daily collected and stored at 14 °C and 70 % RH, were incubated (99.7 °F; 47 % RH) and hatched (99 °F, 43 x 56 x 47% RH) in the partridge farm (room temperature 22 °C and 55 % RH). On day 21, each egg was put inside a cotton gauze bag (15 x 20 cm) to be able to bring together parents and offspring. Each parent group was fed the experimental diets, 30 days before the laying period start. The chemical composition of the experimental diets are shown in the Table 1.

Test - The learning ability and memory retention was tested by the Passive Avoidance Task (PAL) on 144 chicks total (48 chicks for each group). Three one day-old partridge chicks, each coming from a different experimental group, were tested contemporaneously in the same shaving floor cardboard holding box (2 6 x 21 cm), since sociable birds cannot be tested alone (Andrew, 1991). The boxes were continuously warmed by 100 Watt red bulbs (30 cm high) and the room was kept at a constant temperature of 27 °C. Each chick was marked on the head by a different colour, to recognize the different dietary treatment. The chicks were kept in the experimental boxes at least for 30 min prior to the beginning of the trials. The protocol, originally described by Andrew (1991), was partially modified by introducing a pre-training treatment before the passive training, to increase retention levels and reduce variability (Burne & Rogers, 1997). The test consisted of 5 different stages:

Table 1. Ingredients and chemical composition of the diets

| Ingredients (g/kg "as is basis") | Diets Low | Diets Medium | Diets High | Calculated analysis | | Diets Low | Diets Medium | Diets High |
|---|---|---|---|---|---|---|---|---|
| Soybean meal solv extr 44 | 255.0 | 300.0 | 120.0 | Metabolizable Energy | MJ/kg | 10.9 | 11.0 | 11.0 |
| Barley | 234.0 | 160.0 | 70.0 | Crude protein | g/kg | 197.0 | 195.4 | 195.1 |
| Maize grits hominy grits | 150.0 | - | - | Fat | g/kg | 44.9 | 36.2 | 64.9 |
| Soft wheat | 140.0 | 150.0 | 100.0 | Crude fiber | g/kg | 72.4 | 53.1 | 73.3 |
| Maize | 60.0 | 272.0 | 260.0 | Ash | g/kg | 12.6 | 11.8 | 11.6 |
| Calcium Carbonate | 48.0 | 48.0 | 48.9 | Linoleic acid | g/kg | 10.2 | 10.6 | 21.0 |
| Soybean hulls | 33.0 | - | 40.0 | ALA | g/kg | 2.55 | 1.53 | 2.19 |
| Sutured fat supplement | 28.0 | 10.0 | 15.0 | EPA | g/kg | 0.01 | 0.04 | 0.09 |
| Bentonite | 15.0 | 15.0 | 15.0 | DPA | g/kg | 0.10 | 0.68 | 1.46 |
| CaHPO[4] | 15.0 | 15.0 | 10.0 | DHA | g/kg | 0.27 | 1.81 | 3.87 |
| Linseed | 10.0 | 4.7 | - | Total n-3 | g/kg | 2.93 | 4.06 | 7.60 |
| Vitamin and mineral premix* | 4.0 | 4.0 | 4.0 | Total n-6 | g/kg | 10.50 | 14.20 | 25.95 |
| NaCl | 2.3 | 3.0 | 2.9 | n-6/n-3 | 3.58 | 3.50 | 3.41 | |
| L-lysine HCl | 1.7 | 1.0 | 1.5 | | | | | |
| PUFA supplement** | 1.5 | 10.0 | 21.4 | | | | | |
| $NaHCO_3$ | 1.5 | 1.1 | 0.4 | | | | | |
| DL-methionine | 1.0 | 1.2 | 0.9 | | | | | |
| Wheat middling | - | - | 70.0 | | | | | |
| Sunflower seed meal partly decorticated solv extr 35 | - | - | 100.0 | | | | | |
| Soybean whole seeds | - | 5.0 | 120.0 | | | | | |

* Premix supplies (mg/kg diet): retinol 4.5, cholecalciferol 0.075, dl-α-tocopherol 30, menadione 3, thiamin 2, riboflavin 8, pyridoxin 5, cyanocobalamin 0.03, d-biotin 0.1, nicotinic acid 40, pantothenic acid 15, folic acid 1.25, choline chloride 600, Mn 150, Zn 60, Fe 35, Co 0.5, Cu 10, J 0.5, Se 0.1, ethoxyquin 2.5.

** made from Schizochytrium algae

1 - and 2 - chicks were trained to peck a white bead (water bathed bead, 2 mm ∅, about 1 cm in front of the beak) in the pre-training stages;

3 - chicks were trained to peck a red bead and, than, a blue bead (water bathed beads, ∅ 4 mm) in the training stage;

4 - chicks tasted the red bead, bathed in a bitter liquid (methyl anthranilate - MA), in the testing stage;

5 - chicks were checked pecking the red bead and, then, the blue bead in the post-testing stage

Two testers worked together; the first tester presented the beads to the chicks (by a 20 cm long wire, 1 mm cross section), announced the first peck and counted the number of pecks; the second tester, equipped with a chronograph, recorded the elapsed times (latency times), announced the test-end and recorded the number of pecks. Tests lasted 20 s during the pre-training stage and 10 s during the training, testing and post-testing stages. Between each stage and single activity, pauses of different duration were observed. The whole procedure and pause durations are shown in Table 2.

Statistical analysis - All the observed times were converted into their reciprocal values, since original data are not normally distributed. Latencies longer than 20 s during the pre-training or longer than 10 s during training, testing or post-testing stages were considered infinite (reciprocal values equal to zero). The Discrimination Ratios (DR), calculated as blue colour latencies (or pecks number) divided per red colour plus blue colour latencies (or pecks number) (Burne & Rogers, 1997), were previously checked for the normality of their distribution. Latency times and DR were analysed by ANOVA and number of pecks were analysed by the Wilcoxon/ Kruskal- Wallis non parametric test (Rank Sums), in relationship to the n-3 content in the hen diets. The neural development indexes, differences between latencies in the post-testing stage and in the training stage, toward the red bead and the blue bead, were analysed on post-testing crude times, covariated by the training stage times (SAS Institute, 2002).

**Table 2.** Outline of the One-trial Passive Avoidance Task

| Stage | Activity | Stimulus | Interval between trials (min) |
|---|---|---|---|
| 1 | 3 chicks placed in each box | Rest | > 30 |
| 2 | Pre-training (treatment 1) | Chrome bead (2 mm T) + water | 20 |
| | Pre-training (treatment 2) | Chrome bead (2 mm T) + water | 30 |
| 3 | Training (treatment 1) | Red bead (4 mm T) + water | 6 |
| | Training (treatment 2) | Blue bead (4 mm T) + water | 30 |
| 4 | Testing treatment | Red bead (4 mm T) + MA | 30 |
| 5 | Post testing (answering 1) | Dry red bead (4 mm T) | 6 |
| | Post testing (answering 2) | Dry blue bead (4 mm T) | |

## RESULTS

The latency times and the number of pecks are reported in the Table 3. No difference between groups was observed in the pre-training stage. On the other hand, the chicks coming from different groups showed a different vitality in the training stage. Group Medium showed a higher number of pecks given towards the red bead than the group Low ($p<0.05$). Group High did not differ either from Low or Medium. Similar results were observed for number of pecks given towards the blue bead, group High showing a higher number of pecks than the group Low, group Medium not differing either from Low or High. No differences were observed between groups on latency times.

As a consequence of the negative experience (MA-experiencing), in the testing stage a general increment of the latency times and a decrement of the number of pecks given toward all beads (red or blue) was shown by the chicks of every experimental group. However, differences were observed both for latency times and number of pecks in relationship to the different treatments. Chicks of the groups Low and Medium, showed a worse behavioural score than group High, even if the values reached the minimum statistically significant differences ($p<0.05$) only for the blue beads.

## DISCUSSION

These results seem to confirm the possibility of affecting the neural development, the learning ability and the memory retention of the red-legged partridge by means of the parental diet. The n-6/n-3 diet-ratio seems to produce a stronger effect on learning ability (neural chick development index) than n-3 absolute content, in the red-legged partridge too. In this study, with a constant n-6/n-3 diet-ratio (approximately 3.5), the diet containing the low and medium n-3 content gave the worst behavioural scores and, consequently, it was the highest n-3 content (7.60 g/kg) that allowed the better behavioural scores.

Vice versa, in a previous study (Fronte et al., 2008), it was the diet containing the intermediate n-3 fatty acids of 4.04 g/kg and an n-6/n-3 ratio of 3.1, in comparison to ratio with lower and higher n-3 content (n-6/n-3 ratio of 28.6 and 1.5, respectively) that gave the best behavioural score.

Actually, feed producers do not take into consideration the fatty acid composition of the laying diet for game (except for the linoleic acid content) so that, diets apparently equal in the chemical composition, but different in the ingredients, may produce different survival performances of the offspring, after their release into the wild.

The use of laying diets with an n-6/n-3 ratio very far from 3.5, by producing chicks with a reduced neural development, might reduce the survival rate of the offspring and consequently a worse adaptation ability of the red-legged partridges, after their release into the wild.

Table 3. Influence of n-3 content of the hen diet on the latency and number of pecks of the partridge chicks

| Group | | Training stage | | | Post-testing stage | | | Neural development indexes | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | red bead | blue bead | DR* | red bead | blue bead | DR* | diff red | diff blue | diff DR* |
| Latency time (s) | | | | | | | | | | |
| Low | n | 48 | 48 | 48 | 48 | 48 | 48 | 48 | 48 | 48 |
| (2.93 g/kg) | mean | 9.5 | 7.9 | 0.5 | 17.4 | 13.4 A | 0,4 | -7.9 | -5.54 | <0.15 |
| | s.e. | 1.27 | 1.21 | 0.04 | 0.94 | 1.20 | 0.03 | 1.30 | 1.47 | 0.05 |
| Medium | n | 47 | 47 | 47 | 47 | 47 | 47 | 47 | 47 | 47 |
| (4.06 g/kg) | mean | 6.68 | 7.1 | 0.5 | 16.2 | 14.4 A | 0,5 | -9.5 | -7.3 | <0.1 |
| | s.e. | 1.08 | 1.16 | 0.04 | 1.00 | 1.16 | 0.02 | 1.15 | 1.52 | 0.04 |
| High | n | 48 | 48 | 48 | 48 | 48 | 48 | 48 | 48 | 48 |
| (7.60 g/kg) | mean | 6.5 | 5.2 | 0.4 | 15.2 | 9.7 B | 0,4 | -8.7 | -4.5 | 0.1 |
| | s.e. | 1.10 | 1.05 | 0.03 | 1.16 | 1.29 | 0.04 | 1.41 | 1.36 | 0.04 |
| Number of pecks | | | | | | | | | | |
| Low | n | 48 | 48 | 40 | 48 | 48 | 22 | 48 | 48 | 21 |
| | mean | 2.9 B | 2.8 B | 0.5 | 0,4 | 1,0 B | 0.8 | 2.6 | 1.7 | -0.2 |
| Medium | n | 47 | 47 | 44 | 47 | 47 | 20 | 47 | 47 | 18 |
| | mean | 5.2 A | 3.6 AB | 0.4 | 0.9 | 1.3 B | 0.6 | 4.3 | 2.3 | -0.2 |
| High | n | 48 | 48 | 43 | 48 | 48 | 32 | 48 | 48 | 30 |
| | mean | 4.0 AB | 4.8 A | 0.5 | 1.0 | 3.0 A | 0.8 | 3.0 | 1.87 | -0.2 |

Note: means with different letters differ per $P<0.05$. * DR = blue bead value / (red bead value + blue bead value).

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