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Changes in RAPD-DNA Markers and Plasma Protein Profile in Progesterone-Treated Chicken

Tito Naeem Habib[1], Gamal El-Din S. Amin[1], Ahmed Ahmed Hussien[1], Mohammed Salama[2], Mohamed Omar Ahmed[1], Motamed Elasyed Mahmoud[3,*]

[1]Molecular Genetics Lab, Department of Zoology, Faculty of Science, Sohag University, 82524, Egypt
[2]Department of Zoology, Faculty of Science, Ain Shams University, 11566, Egypt
[3]Department of Animal Behavior and Husbandry (Genetics, Breeding and Production), Faculty of Veterinary Medicine, Sohag University, 82524, Egypt
*Corresponding author: motamed71111@gmail.com, moatamed_ali@vet.sohag.edu.eg

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Abstract The abuse of contraceptive pills as growth promoters in the poultry industry has serious health concerns. Consumption of hormonal residues in animal products results in reproductive disorders and an increased risk of cancers in humans. This study aimed to investigate the effect of treatment with progesterone hormone on the plasma proteins and RAPD-DNA markers of broiler chickens. One hundred, one-day-old broiler chicks were equally divided into 5 groups (n = 20). Four groups were treated daily with progesterone hormone either subcutaneously or orally (1.0 mg, 1.5mg/kg). The 5th group was control non-treated chicken. Sodium dodecyl sulfate polyacrylamide gel-electrophoresis (SDS-PAGE), and RAPD assays in conjunction with productive parameters such as growth rate were assessed. SDS-PAGE and RAPD-DNA patterns were significantly affected by progesterone hormone treatment. Either or oral or subcutaneous progesterone hormone treatments increased the body weight of chicks compared to control. Treatment with progesterone hormone induces effects on both DNA and plasma proteins in broiler chickens. Changes at the genomic DNA level may be due to specific modifications in RAPD patterns or arise as a consequence of hot spot DNA changes and/or mutations.

Keywords: *Xeno-progesterone, plasma protein profile, RAPD-DNA markers, growth rate, broiler chicken*

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1. Introduction

Naturally occurring hormones, such as progesterone, oestrogen, and testosterone, are necessary for various physiological processes in animals and humans. These chemicals are excreted in urine and feces as metabolites or conjugated forms, leading to contamination of water, milk, and animal product, which are consumed directly or indirectly by humans, plants, and animals [1]. The carcinogenic and endocrine-disrupting potentials are the main health concerns associated with the use of hormonal compounds as growth promotants or therapeutic agents. Certainly, poultry, cow and horse manure contains the greatest amount of steroidal estrogens that may contaminate the environment [2]. Several reports confirm that diethylstilboestrol endangers the health of animals and humans when repeatedly used in large doses. Further, cooking or frozen storage did not affect the nature or quantity of their metabolites [3].

The six hormonal types most widely used in meat production include three natural hormones, oestradiol 17, testosterone, and progesterone, and three synthetic substances, trenbolone, zeranol, and melengestrol acetate [4]. Although some studies indicated that estradiol-17 beta has the genotoxic potential [2]. Scientists of Joint FAO/WHO Expert Committee on Food Additives pointed out that no data are demonstrating that concentrations below the no-hormonal-effect level cause adverse effects in animals or humans [5]. Under their normal biochemical action, low concentrations of steroid hormones (nM) bind to and activate their intracellular receptors, which interact with hormone response elements in DNA, leading to the transcription of genes that induce cell proliferation and growth [6]. Therefore, a hormonal substance could promote carcinogenicity in hormone-sensitive tissues through such a proliferative mechanism [7,8]. Though receiving less attention, the other health concern is the endocrine-disrupting effects of hormonal growth promotants, notably their significant potential to perturb normal development in sensitive subpopulations like prepubertal children and developing fetuses [9].

Only limited new information was presented in the European Commission studies and other cited literature regarding the metabolism, endocrine-disrupting potential,

genotoxicity, or carcinogenicity of progesterone [10]. Studies on the potential environmental impact of hormonal growth promotants were considered to have limited value in assessing the dietary risk of hormonal residues to humans [11]. A study found that a chicken sample from a local market to have a higher than normal level of estrogen when compared to beef samples [10]. Also, residues of zeranol were reported in the blood of some of the girls who had reached puberty early [12,13]. That would be due to the abuse of hormones [14].

The present study is concerned with reporting the changes in plasma proteins and RAPD-DNA profiles that reflect gene expressions and DNA effects and compared to fitness parameters in chicken exposed to treatment with progesterone subcutaneously and orally.

2. Material and Methods

2.1. Animals

One hundred a day-old Ross-broiler chicks were purchased from El-Diabat poultry farm, Sohag, Egypt. All birds were reared according to the guidelines of Sohag University Ethical Committee (Approval number: Sohag, 02/04/2020/01). The feed was obtained from the same farm supplier company (Alsalam Feed, Assiut, Egypt). Components contain about 16% raw protein, 3.21% fats, and 3.01% fibers. Chicks were injected with 1 mL of Newcastle disease virus (NDV; killed vaccine, Formosa Biomedical Inc., Taipei, Taiwan) into the leg muscle at 5th week of age.

2.2. Progesterone Hormone Treatment

According to the previous studies that determined the appropriate dose of growth hormones empirically, the administered daily doses of medroxyprogesterone acetate (Provera, Pfizer, Egypt) were either 1 or 1.5mg/kg, for both subcutaneous and oral routes [15].

2.3. Plasma Proteins and Gradient Gel Electrophoresis

Peripheral blood was collected from the wing vein into heparinized tubes, centrifuged at 3000g for 20 min at 4℃ and the plasma was stored at 20℃ as described by Berry et al. [16]. Gradients (10-20%) of sodium dodecyl sulfate, polyacrylamide gel-electrophoresis (SDS-PAGE) were formed by running the gel solution from a gradient mixer down one edge of the gel slab using BIO-RAD (Protean Ii Xi) Cell. The gel was electrophoresed for 4 hours at 15 V/cm until the bromophenol blue reaches the bottom of the resolving gel. The gels were fixed, stained with Coomassie Brilliant Blue R-250. To make a permanent record of plasma protein profiles, the stained gel was photographed and dried [17].

2.4. DNA Isolation and RAPD-PCR

Genomic DNA was isolated from blood samples of each individual using the phenol-chloroform isoamyl alcohol method [18]. DNA used in these reactions was diluted to 50 ng/µl. Each RAPD reaction mix contained 2 mM MgCl2, 10X PCR buffer, 100 M dNTPs, 1.25 units of Taq DNA Polymerase (Promega Corporation, USA), genomic DNA, a primer, and water in a total volume of 25 µl, and Oligonucleotide concentration of 100 pmol/25 l with 50 ng of genomic DNA. DNA thermal cycler (Perkin Elemer) was used for the reaction run. Denaturation was performed at 95℃ for 3 min followed by the addition of Taq DNA polymerase. RAPD primers (OPD1-20) were purchased from Operon Technologies (Alameda, CA, USA). Table 1 represents the list of sequences that showed repeatability of results.

Table 1. List of single primers (octamer) sequences

Octamer	Sequence
OPD12	CACCGTATCC
OPD13	GGGGTGACGA
OPD15	CATCCGTGCT
OPD16	AGGGCGTAAG
OPD20	ACCCGGTCAC

The reaction conditions were 1 cycle of 94℃ for 5 min, 37℃ for 2 min, 72℃ for 1 min, 25 cycles of 94℃ for 1 min, 37℃ for 2 min, 72℃ for 3 min, 1 cycle of 94℃ for 1 min, 37℃ for 2 min, and 72℃ for 10 min. The PCR amplified bands were separated by electrophoresis on 1.5% agarose gels, stained with ethidium bromide, and scored visually for band presence or absence. PCR reactions were repeated on the same sample subset at least two times for reproducibility.

2.5. Statistical Analysis

Analyses of plasma protein fractions and RAPDs bands were assessed by comparing the means of each molecular assay in different chicken groups, using GenePop Software Version 2.1 [19] program. Similarity values were converted into genetic distance using the formula: D =1S. Similarity coefficients between different chicken groups were calculated according to Nei and Li [20] and lynch [21]. Bodyweight data for each age group were analyzed by one-way ANOVA and turkey's post hoc test using Graph Pad Prism software.

3. Results

3.1. Plasma Proteins' Analysis

Results in Figure 1 showed complete deletion of the pre-albumin protein (band 2) from all treated groups with a high molecular weight of 147 kDa, and a partial deletion related to the first and second orally-treated groups (band 3) with 135 kDa ($P< 0.05$). Regarding, the post-albumin proteins, there was a distinct addition of a new band (band 16) in all treated groups with the lowest molecular weight of 27 kDa ($P< 0.05$). Also, there was a complete addition and deletion in the protein fraction (bands 10 and 15) in all treated and external control groups (obtained from the market) with a molecular weight of 57 kDa and 29 kDa, ($P< 0.05$), respectively.

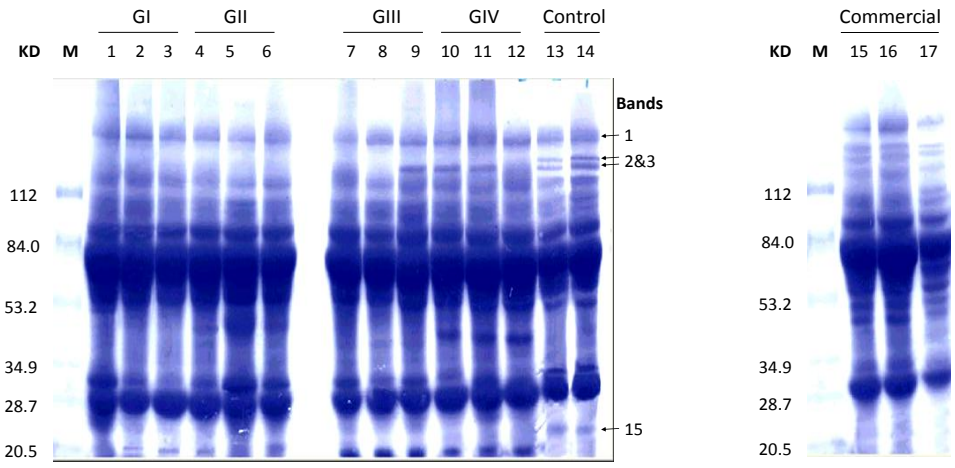

Figure 1. (Color online) SDS-PAGE plasma protein profiles of broiler chicken exposed to 1mg and 1.5 mg subcutaneous, and 1.5 mg oral administrations of Medoroxy-progesterone acetate/kg daily for 3 weeks. The molecular sizes in kilo Daltons (kDa) of selected bands are indicated on the left of each gel; group I (1.0 mg/kg, subcutaneously, lanes, 1, 2, and 3), group II (1.5 mg/kg, subcutaneously, lanes, 4, 5 and 6), group III (1.0 mg/kg, orally, lanes, 7, 8 and 9), group IV (1.5 mg/kg, orally, lanes, 10, 11 and 12), group V (control, lanes, 13 and 14) and commercial chicken; lanes 15, 16 and 17. M= Bio-Rad protein markers (low range)

Based on different electrophoretic bands regardless of their intensities, it was able to determine 5 unique profiles for plasma proteins compared with the profile of the control group, while the remaining samples can be classified into four patterns; group I (1.0 mg/kg, subcutaneously injected) with 2 deficient bands (2 and 15) were detected, group II (1.5 mg/kg, subcutaneously) showed absence of 3 bands (2, 12 and 15); groups III and IV (orally-treated groups) with the addition of 2 bands were observed, and the commercial group with the absence of 2 bands (15 and 16). The differences in some bands' intensities may be reflected as differences in gene expression.

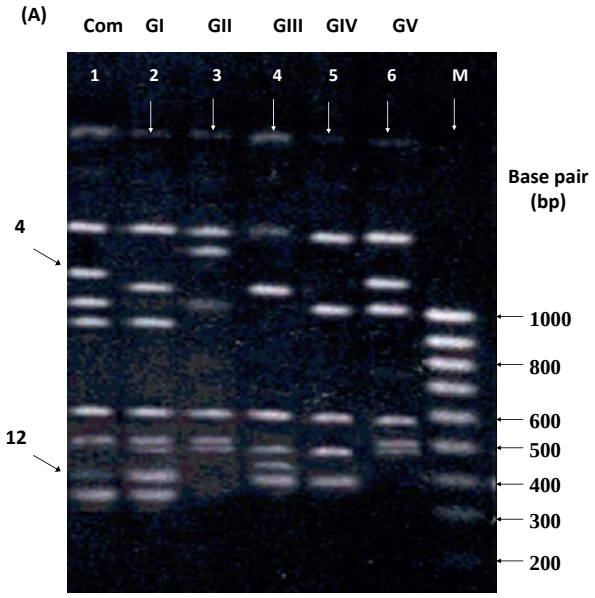

Figure 2. Gel electrophoretic image represents an image for RAPD profiles produced by using OPD12 primer. The molecular sizes in base pairs (bp) of selected bands are indicated on the left of each gel; lane 1 Com.= represents commercial chicken; lanes from 2- to 5 for progesterone treated groups [lane 2 = GI (1mg, subcutaneous); lane 3= GII (1.5 mg/subcutaneous); lane 4 = GIII (1 mg orally); lane 5= GIV (1.5 mg, orally) 2nd orally], and; exposed to either 1mg, or 1.5 mg/kg orally on daily basis for 3 weeks and lane 6 for control non-treated chicken. DNA control; and lane M= 100 bp DNA ladder

RAPD-DNA profile generated by primer OPD12 showed the disappearance of band 4, with higher molecular weight (band 4; 1339 bp) while band 12, of low molecular weight (band 12; 368 bp) appeared in all exposed chickens higher than control ($P < 0.05$, Figure 2 A). Similarities among the different groups were estimated using a distant matrix of scored bands (Figure 2).

The RAPD profile generated by primer OPD12 showed a simple matching coefficient of similarity ranged from 0.75 for the most closely related hormonal-exposed groups to 0.57 for the most distantly related ones (hormonal-free one, Figure 3).

However, it was clear that some bands followed a specific pattern of variation in both progesterone-treated and control chickens groups which were perturbed to be dependent on the time of exposure. This phenomenon was also observed for some other bands (Figure 2).

Based on simple matching coefficients and accessions chicken can be divided into high and moderate affected groups. Orally affected accessions showed RAPD-profile which were genetically very close, with some exceptions. The three hormonal-disruptive groups are placed very far from the control, indicating very diverse hormonal effects; similarly with commercial accessions; samples from the markets, which is presumably material, are quite separate and there are unique RAPD patterns in the two exposed ones. Between the hormonal-treated groups (orally and subcutaneously), there is a distinct deviation from the control one (Figure 3).

The extent of variations seemed to depend on the choice of primer. Primer OPD13, OPD15, and OPD16 showed variation in RAPD profile observed in both exposed and control groups, whereas for primer OPD20 displayed stable and little variations between the progesterone-treated and commercial groups when compared with controls profiles (Table 2).

Among these two orally treated groups (Table 2), is the most distant from control, which shows the highest degree of affinity, followed by samples of the subcutaneous. Surprisingly, the samples from the commercial markets are also far apart from the control. The commercial group

is composed of some different broods showing a high degree of similarity and the other more distant hormonal-treated groups (Table 3).

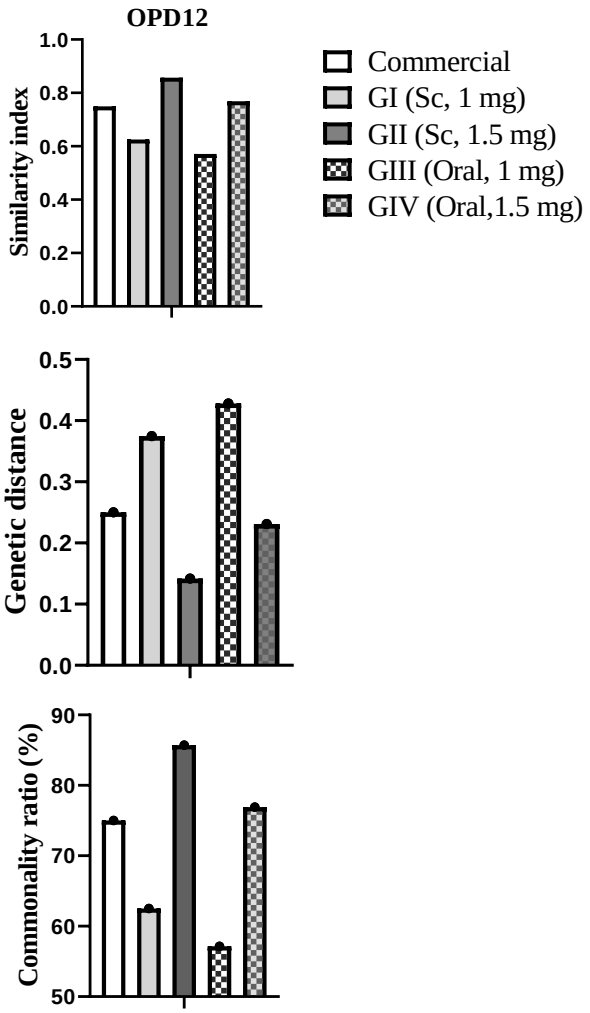

Figure 3. The similarity coefficient, genetic distance, and commonality indices produced by primer OPD12 in progesterone-treated chicken compared to the control

Table 2. Genetic distance in progesterone-treated, commercial and control chicken

Groups	Com	Subcutaneous			Oral
Octamer	None	GII (1 mg)	GIII (1.5 mg)	GIV (1 mg)	GV (1.5 mg)
OPD12	0.25	0.38	0.14	0.43	0.23
OPD13	0.10	0.09	0.38	0.11	0.11
OPD15	0.19	0.151	0.14	0.08	0.08
OPD16	0.16	0.076	0.08	0.33	0.23
OPD20	0.26	0.11	0.11	0.11	0.11

Abbreviations: Com, represents commercial chicken; groups from 2- to 5 for progesterone (PH) treated groups [GII = Group II (PH 1mg/kg, subcutaneous); GIII = Group III (1.5 /kg, subcutaneous); GIV = Group IV (1 mg/kg, orally); GV= Group V (1.5 mg /kg, orally)].

From the present data in Figure 4, it seems that during the first 20 days, the average body weights of the two experimental chicken groups (after orally and subcutaneously, PH treatment), was duplicated to reach its (118%) and in an oral group (92%) compared to control as (58%) After that, the growth rate was somewhat elevated to about 75% control group, and more accelerated as in both oral and in the subcutaneous one. Finally, the rate of growth was higher in all progesterone-treated groups compared to the control.

Table 3. Similarity index in progesterone-treated, commercial, and control chicken

Groups	Com	Subcutaneous			Oral
Octamer	none	GII (1 mg)	GIII (1.5 mg)	GIV (1 mg)	GV (1.5 mg)
OPD12	0.75	0.61	0.84	0.56	0.76
OPD13	0.89	0.91	0.61	0.88	0.87
OPD15	0. 80	0.83	0.84	0.91	0.91
OPD16	0.81	0.91	0.91	0.65	0.75
OPD20	0.73	0.87	0. 88	0. 88	0. 88

Abbreviations: Com, represents commercial chicken; groups from 2- to 5 for progesterone (PH) treated groups [GII = Group II (PH 1mg/kg, subcutaneous); GIII = Group III (1.5 /kg, subcutaneous); GIV = Group IV (1 mg/kg, orally); GV= Group V (1.5 mg /kg, orally)].

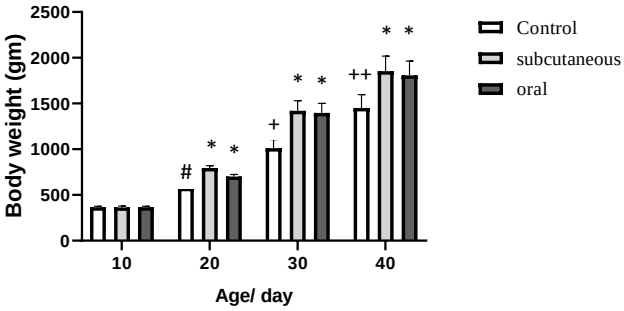

Figure 4. Effect of subcutaneous and oral progesterone treatments on body weight of broiler chicken. Body weights were measured in grams at 10, 20, 30, and 10 days old. Data were presented as means ± SD, only values with asterisks as superscripts were significant if $P < 0.05$, versus the control group

4. Discussion

Despite growing concern over the presence of genotoxins in our environment, there is a lack of knowledge about the potential consequences of DNA damage and mutations concerned on a public health level. In this study, changes in plasma proteins and RAPD profiles that reflect gene expression and DNA effects were compared to fitness parameters in broiler chicken exposed to progesterone hormone concentrations via two different metabolic pathways; subcutaneous and oral ways of injections. Plasma proteins and DNA effects include bands disappearance/appearance and DNA damage as well as mutations and possibly other effects at the DNA level, which can be induced by the hormonal disruption that may directly and/or indirectly, interact with genomic DNA.

The present results showed a recovery of some protein fractions after 3 weeks of exposure in both exposed chicken groups (orally and subcutaneous). For example, the recovery of post-albumin protein fraction, with MW of 39.6 kDa indicates that the formation of albumin adducts should be investigated as a potential marker of exposure. These protein fractions are available in the plasma for reaction with reactive metabolites without the need for the metabolites to cross the cellular membrane, where small exposure (sub-chronic) can occur daily. Since the metabolism of the main mass of blood plasma proteins is under hormonal control and their synthesis occurs predominantly in the cells of the liver and lymphoid

system [22]. The end-of-shift measurement of albumin adducts would yield a good and sensitive marker of exposure.

The effect of deletion caused by the hormonal treatment may be considered as a successfully distinguished fingerprint of progesterone disruption after 3 weeks of exposure. The previous data are in agreement with the results obtained by Bergeron [23], who concluded that progesterone inhibits the synthesis of specific receptors located in the nuclei of epithelial and stromal cells of the endometrium. The appearance of a new post-albumin fraction 40 kDa (band 12), due to progesterone treatment on blood plasma proteins and after 3 weeks was lessened gradually and became near to control. A similar finding was reported by Gritsuk [22]. He found out that the level of alpha-1 globulins in rats reduces on Days 3, and 10, increases on Days 30, and 70, and normalizes on day 140 of exposure. Thus, in this case, there is an ability of the body to adapt to the hormonal effect through either its' regulation within the liver or excretion by the kidney or related to the immunosystem of the body.

The RAPD-DNA assay has been successfully used to monitor the presence of DNA effects, including DNA damage and potential mutations, induced by diverse genotoxins. Recently, this method allowed detection of benzo (a) pyrene, copper [24-26], mitomycin C, ultraviolet [27], Verrucarin J, and Aflatoxin B1 toxins [28,29] induced DNA effects, in aquatic and terrestrial systems. The most significant advantages of the RAPD-DNA technique lie in its speed, applicability to any organism, and potential to detect a wide range of DNA damage and mutations (including point mutations and large rearrangements). For example, a set of experiments performed *in vitro* revealed that DNA alterations such as DNA breakage, benzo(a)pyrene adducts, and thymine dimers as well as a mutation in the sequence of the primer induced significant changes in RAPD profiles [27,30]. However, the RAPD-DNA assay allows a qualitative assessment of the DNA effects and the nature of the changes in profiles can only be speculated unless amplicons are analyzed (e.g., sequencing, probing, etc.). Although DNA damage and mutations seem to be the main factors influencing RAPD profiles, other factors such as variation in gene expression, steady levels of genetic alterations, and changes in metabolic processes may also induce some changes in RAPD patterns [25].

In this study, the potential use of the RAPD technique for the detection of DNA effects induced by progesterone disruptions on chicken DNA was demonstrated. The use of two DNA concentrations confirmed that the changes occurring in RAPD profiles were due to true polymorphisms rather than a false amplification [31] as reproducibility was excellent. Modifications of the RAPD patterns are likely to be due to one or a combination of the following events: (i) changes in oligonucleotide priming sites due mainly to genomic rearrangements and less likely to the DNA damage and point mutations in the primer binding sites because the binding site is only 10 base long whereas genomic rearrangements occur to much larger fragments (e.g., several Kb), (ii) structural changes owing to DNA damage and less probably to mutations because the constraint-induced by DNA damage such as DNA adduct is much important than a point mutation, and (iii) interactions of the *Thermus aquaticus'* DNA polymerase with damaged DNA [24,26,28]. Also, (iv) cell physiology, which is known to have a direct influence on DNA may also affect RAPD profiles [32].

The common changes occurring in RAPD profiles may not be attributed to the covalent binding of the progesterone hormone to the DNA molecule at the same site(s) because of the structural differences between the two compounds. An alternative and more plausible explanation is that progesterone and/or their metabolites interact with the same compound(s), possibly after binding to the receptor, which then induces the same effects at the DNA level. These intermediates could bind directly to the DNA and thus create identical adducts. Indeed, Liehr et al. [33] demonstrated that structurally diverse estrogens (e.g., E2, diethylstilbestrol) covalently modified nucleotides in the target tissue. Besides, estrogens are known to induce numerical chromosomal changes (aneuploidy) and structural chromosomal aberrations [34].

RAPD markers can detect mutations if they occur in at least 2% of the cells [28]. In a similar titration experiment, comparable results were obtained with the limit of detection estimated to be less than 5% [28]. By extrapolation, this means that DNA damage can also be detected if they occur in at least 2% of the cells. In this context, the results obtained in the present work suggest that hot spot DNA effects (e.g., DNA damage and/ or mutations) occurred in at least 2% of the cells for chickens exposed to progesterone hormone at low concentrations. It has been reported that metabolites of estrogen, termed catechol estrogen quinones, can form DNA adducts and cause oxidative DNA damage (ODD) [35]. This is well in agreement with the present study.

The measure of some fitness parameters (e.g., weight and/or growth rate) and genetic makeup present several advantages. First of all, in ecotoxicology, it is fundamental to accumulate data at different levels of biological organizations to fully understand the effect of a given toxicant on an organism [26]. Secondly, the measure of some parameters at the fitness level facilitates the interpretation of the data at the molecular level. In the present work, a significant increase in growth rate correlated with a significant stimulation in DNA replication during the first 3 weeks of hormonal treatment. After that, a significant reduction in growth was detected in the subcutaneous group and continued to be similar in the oral group compared to controls. Thus, growth in the subcutaneous group correlates with significant inhibition in DNA replication, suggesting that the extent of DNA damage may be important effect in the majority of the cells, while in the oral group, it could be assumed that DNA damages were efficiently repaired and that DNA replication was not significantly inhibited due to the diluted treatment. During the period (30-40 days) of exposure, it was noticed a distinct depletion in the growth rates of both experimental groups compared to the neutral effect occurring in controls. This could help to explain how xeno-progesterone mimics the effects produced by natural progesterone. A corresponding finding on the DNA toxicity induced by 17p-estradiol and low concentrations of 4-nonylphenol are thought to cause endocrine disruption [36,37].

5. Conclusion

Plasma proteins footprint and DNA damage as indicated in SDS-PAGE and RAPD analysis assays, respectively, suggest that progesterone hormone may induce common DNA effects in white broiler chickens. The present investigation suggested that changes at the DNA level may be the precursors of some of the numerous effects reported at higher levels of biological organization such as the feminization of males, developmental abnormalities, tumorigenicity, and infertility regarding public health.

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Author Contribution Statement

T.N.H., M.S., and M.E.M. designed the study. G.E.S.A. and T.N.H. performed the experiments. All authors have read and approved the final version of the manuscript.

Disclosure Statement

All authors disclose the absence of any type of interest conflict.

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