The Clamshell and Fishbone Can Increase Thyroid Hormones Effectiveness to Improve Muscle Strength

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Abstract. Clamshells contain aromatase inhibitors that can increase testosterone, while fishbone can be an effective animal protein source. Bangkok rooster has an impressive muscle characteristic and physical activity that indicates manifestations of testosterone. T3 and T4 are thyroid hormones that mediate the conversion of proteins into energy. Bangkok roosters demand muscle strength to support their performance and physical activity. This study aimed to determine the influence of clamshell and fishbone on T3 and T4 levels and muscle structure. Three-month-old Bangkok roosters underwent a 35 days experiment. All animals consisted of three groups: P0 ($n = 6$) did not receive any treatment, P1 ($n = 6$) received shellfish and fishbone treatment, and P2 ($n = 6$) only received fishbone treatment. The blood samples were collected once per seven days of the experiment. Then, enzyme immunosorbent assays had applied to analyze weekly serum T3 and T4. Euthanasia was done on the 35th day to collect muscle specimens. Finally, histological preparation proceeded for each pectoral muscle specimen to calculate muscle fasciculus size. The 35 days trial showed a T3 reduction ($p < 0.05$), a T4 elevation ($p < 0.05$), and a fasciculus widening ($p < 0.05$) in the P1 rooster. This study concluded that the provision of clamshell and fishbone significantly affected T3 and T4 levels and enlarged the area of muscle fasciculus.

Keywords: Aromatase blocker · *bangkok rooster* · *muscle fasciculus* · *T3* · *T4*

1 Introduction

Modernization in poultry produces chickens with an exceptional growth rate. The rapid growth of chicken requires state-of-the-art husbandry to manage optimum production. Feed advancement is part of precision husbandry that can optimize chicken productivity. Study on feedstuffs not only improves productivity but also contributes to sustainable agriculture.

J. Sumantyo et al. (Eds.): ICoSIA 2022, ABSR 29, pp. 360–368, 2023.
https://doi.org/10.2991/978-94-6463-122-7_34

Clamshell and fishbone are natural materials that appear as the byproduct of aquaculture and marine industries. The abundance of clamshell and fishbone may substitute and conserve the availability of chicken feedstuff. High zinc content in clamshell expresses an aromatase blocker effect that boosts testosterone activity [1, 2]. Fishbone contains a high protein fundamental for muscle improvement [3, 4]. The muscular androgen receptor will react with testosterone which arouses myofiber proliferation and protein synthesis [5, 6]. The androgenic activity in muscle can improve chicken productivity. The clamshell action is safer than the exogenous androgen. Exogenous testosterone directly risks the reproductive endocrine system in humans and animals [7]. Because extra testosterone consumption severely suppresses gonadotropin secretion. Aromatase inhibition of the clamshell increases testosterone endogenously. Endogenous testosterone develops physiologic suppression that is tolerable to maintain the reproductive system. Therefore, clamshells and fishbone are desirable to increase chicken productivity through testosterone elevation and muscle performance improvement.

The thyroid is a gland that releases triiodothyronine (T3) and thyroxine (T4). The secretion of thyroid hormones is under the control of thyroid-stimulating hormone (TSH) from the anterior pituitary. The T3 and T4 activities maintain fat and protein that influence body weight. T3 and T4 stimulate the energy extraction from protein and fat for physical activity or other body functions [8, 9]. Exogenous T3 and T4 treatments minimize body weight gains in broilers [8]. Chronic thyroxine treatment reduces body weight and enhances blood glucose levels in broilers [9]. The aromatase blocker effect of clamshell on T3 and T4 requires further study because of its impacts on muscle protein.

The muscle is a significant organ in whole-body protein metabolism. Therefore, an additional protein intake can affect the muscle structure in chickens. An extra protein ration in seven-day-old Kampung chicken produces sounder pectoralis muscle development [10]. The protein-rich attribute of the fishbone is a prospective protein source to enhance muscle performance. The T3 and T4 activities regulate muscle performance because of the protein degradation effect to meet energy demand. A higher T3 and T4 treatment improves pectoral muscle weight but reduces abdominal fat in the broiler [8, 11]. The protein breakdown rate becomes faster as the T3 and T4 escalate [8]. The study of T3 and T4 is crucial to understand muscle protein control.

Bangkok rooster has superior muscle strength due to its fighting cock origin in Thailand [12]. The Indonesian people hybridize the Bangkok chicken for diverse utilization, including meat production [13, 14]. Broilers have a vigorous genetic selection to achieve rapid muscle growth, which modifies muscle characteristics [15–17]. Bangkok rooster promisingly becomes an excellent meat-producing chicken with a unique meat characteristic. The investigation of clamshell and fishbone alterations on muscle is necessary for the Bangkok rooster.

The demand for protein sources urges the poultry industry to build precision husbandry. The promising potency of clamshell and fishbone to boost productivity can be crucial in poultry. T3, T4, and muscle are fundamental to understanding chicken productivity. This research intended to evaluate the effect of clamshell and fishbone on T3 and T4 profiles and muscle fasciculus area in Bangkok roosters.

2 Materials and Methods

2.1 Animal

This study employed 18 three-month-old Bangkok roosters (*Gallus gallus*), which originated from an ornamental chicken breeder in Bantul Regency, Yogyakarta Special Region, Indonesia. The maintenance and treatments took place in Bantul Regency, Yogyakarta Special Region, Indonesia, from February to March 2022. The rooster housing method was semi-intensive, with individual platform wooden chicken houses and slit bases. All processes in this study referred to the standard from the Ethics Committee of the Integrated Testing and Research Laboratory Universitas Gadjah Mada (LPPT UGM) with certificate number 00009/04/LPPT/III/2021.

The study design in this research was Randomised Control Trial (RCT) method. Three rooster groups undertook the experiment for 35 days. Each treatment group comprised six animals that consumed treatment powder. The treatment groups were P0: without treatment; P1: fishbone 3.3 g/day + clamshell 6.6 g/day, and P2: fishbone 3.3 g/day. The fishbone originated from brackish water milkfish (*Chanos chanos*). Atomic absorption spectrometry (AAS) detected 35.75% protein in the milkfish thorns. The clamshell contained approximately 61.55 mg/kg of Zn, according to the previous study [1]. Fishbone also provides several amounts of zinc, particularly 0.59 mg/100g in tuna and 1.40 mg/100g in sea bream [18, 19]. The feed provision was 150 g/rooster twice daily with ad libitum water for all roosters.

2.2 Serum T3 and T4

The serum T3 and T4 were analyzed using an Enzyme-linked immunosorbent assay (ELISA) according to the manual (Calbiotech T4 ELISA T4224T and T3 ELISA T3379T, USA). The sera from days 0, 7, 14, 21, 28, and 35 of the experiment were sampled and stored at -20 °C for analysis.

2.3 Hematoxylin-Eosin Staining

Hematoxylin-eosin (HE) staining method could measure the fasciculus area from a muscle specimen [20]. The pectoral muscle was chopped into 1x1 cm and fixed with 10% neutral buffer saline (NBF). Subsequently, a 5-micron trimming proceeded using microtome after paraffin embedding. Finally, the histological image was collected using a light microscope (Leica, Germany) with a digital camera system. The fasciculus area calculation was computational using ImageJ (National Institute of Health, USA) software.

2.4 Statistical Analysis

The statistical analysis employed one-way ANOVA at a 95% confidence level ($\alpha = 0.05$) with SPSS 16.0 software. Then, the Duncan test complemented the ANOVA to identify the different values.

3 Results

Table 1–2 presented the T3 and T4 profiles in Bangkok roosters after 35 days of treatment. The T3 and T4 examinations subjected serum samples to the ELISA method. All roosters had shown no significant changes in T3 and T4 profiles during 0 – 28 days of treatment ($p > 0.05$). The roosters in P1 presented an elevation of T4 levels on the 35th day of treatment ($p < 0.05$). These results indicated that clamshell and fishbone could influence T3 and T4 profiles after 35 days of treatment.

Table 3 listed the muscle fasciculus areas in Bangkok roosters that showed the effect of the treatments. The fasciculus area originated from a pectoral muscle specimen. First, the pectoral muscle samples underwent histological preparation and staining. Afterward, ImageJ software assisted in the fasciculus area calculation computationally. Bangkok rooster in P1 displayed the broadest fasciculus area, while P0 was the tiniest ($p < 0.05$). These findings indicated the effect of clamshells and fishbones on muscle structure.

Table 1. Serum T3 levels of Bangkok rooster under 35 days of treatment

P	Mean±SD of T3 (ng/mL), days -					
	0	7	14	21	28	35
P0	2.08 ± 0.08^{a}	1.85 ± 0.13^{a}	1.79 ± 0.66^{a}	1.92 ± 0.68^{a}	2.27 ± 1.09^{a}	2.34 ± 0.39^{a}
P1	1.92 ± 0.16^{a}	2.05 ± 0.51^{a}	1.72 ± 0.67^{a}	1.75 ± 0.65^{a}	2.01 ± 0.42^{a}	1.77 ± 0.41^{b}
P2	2.27 ± 0.48^{a}	1.84 ± 0.37^{a}	2.02 ± 0.55^{a}	2.35 ± 0.30^{a}	2.03 ± 0.33^{a}	2.65 ± 0.39^{a}

[a,b] Different letters in the same column show significant differences ($p < 0.05$). P0: control, P1: fishbone 3.3 g + clamshell 6.6 g, P2: fishbone 3.3 g. P: treatment, SD: standard deviation.

Table 2. Serum T4 levels of Bangkok rooster under 35 days of treatment

P	Mean±SD of T4 (μg/dL), days -					
	0	7	14	21	28	35
P0	2.06 ± 1.08^{a}	1.98 ± 0.61^{a}	1.06 ± 0.15^{a}	1.42 ± 0.37^{a}	1.24 ± 0.45^{a}	1.29 ± 0.14^{b}
P1	1.33 ± 0.05^{a}	1.81 ± 0.93^{a}	1.21 ± 0.31^{a}	1.22 ± 0.19^{a}	1.13 ± 0.07^{a}	1.62 ± 0.25^{a}
P2	1.22 ± 0.24^{a}	1.15 ± 0.23^{a}	1.07 ± 0.20^{a}	1.27 ± 0.52^{a}	1.34 ± 1.01^{a}	1.11 ± 0.21^{b}

[a,b] Different letters in the same column show significant differences ($p < 0.05$). P0: control, P1: fishbone 3.3 g + clamshell 6.6 g, P2: fishbone 3.3 g. P: treatment, SD: standard deviation.

Table 3. Fasciculus area of Bangkok rooster after 35 days of treatment

P	Means ± SD of fasciculus area (μm^2)
P0	637975,74 ± 19100,16[c]
P1	1088556,12 ± 110030,80[a]
P2	815561,77 ± 38518,41[b]

[a,b,c] Different letters in the same column show significant differences ($p < 0.05$). P0: control, P1: fishbone 3.3 g + clamshell 6.6 g, P2: fishbone 3.3 g. P: treatment, SD: standard deviation.

4 Discussion

Clamshell possesses an aromatase blocker attribute due to its high zinc content. The Zn transporter transfers blood zinc into the intracellular zone [21]. Intracellular zinc phosphorylates cellular proteins as a second messenger [21, 22]. The intracellular phosphorylation of aromatase blocks the testosterone–estradiol conversion [23]. Therefore, aromatase blockers elevate androgen activity by hindering the conversion of testosterone into estradiol. Testosterone can cause muscle hypertrophy in humans and animals. The androgenized female rats show enlargement of the gastrocnemius and tibialis anterior muscles [24]. Furthermore, exogenous testosterone has been widely effective for muscle-wasting medication in humans [25–27].

The aromatase blocker strengthens testosterone activity, which produces muscle hypertrophy. Testosterone interacts with the muscular androgen receptors (AR), which proceeds DNA-binding dependent and non-DNA-binding dependent signaling pathways [28]. The DNA-binding dependent action of testosterone begins with the translocation of the androgen-AR complex into the nucleus. Then, the androgen-AR complex binds to DNA to regulate the transcription process. The androgen non-DNA-binding dependent pathway involves second messenger activation and ERK, Akt, and MAPK molecules. Testosterone stimulates Akt to activate mTOR, which induces muscle growth and protein synthesis [6]. Clamshell and fishbone ingestion stimulates muscle growth via testosterone elevation and protein synthesis.

The results suggested that clamshell and fishbone consumption enlarged the fasciculus area in P1. The fishbone treatment boosts dietary protein in the Bangkok rooster. A high-protein diet and high testosterone activity promote protein deposition in muscle. Protein deposition opposes T3 and T4 functions that trigger protein breakdown [8]. The administration of testosterone depresses circulating T3 in chickens [2, 29]. Amphibians exhibit T3 reduction and metamorphosis disruption due to exogenous testosterone [30]. However, P1 disclosed serum T4 elevation and T3 depletion. T4 can become a prohormone to generate serum T3 under deiodinase regulation in the peripheral tissues [31, 32]. The mild serum T3 levels in the blood promote TSH production to drive T4 secretion from the thyroid gland [32, 33]. The high blood testosterone activates the thyroid gland to secrete predominantly T4 and a minor amount of T3 into circulation [34]. Besides, testosterone also stimulates hepatic deiodinase 1 (dio1), which metabolizes serum T4 and T3 [34, 35]. Dio1 poorly catalyzes T3 but strongly metabolizes sulfated-T3 (T3S)

to recollect iodothyronines [32]. High testosterone levels in Bangkok roosters probably induced thyroidal T4 secretion and hepatic T3 degradation.

Physiological energy expenditure increases T3 activity from the liver, muscle, and adipose tissue [32, 36]. Food availability increases blood glucose levels which triggers insulin to promote T4 conversion into T3 in peripheral tissues [32]. Thyroid hormones can also stimulate the proliferation of satellite cells and the maturation of muscle cells [11]. Efficient energy expenditure in the P1 rooster might indirectly cause the peripheral tissues to withhold T3 release in circulation. The low circulating T3 shifts energy usage into energy deposition, which favors protein synthesis and muscle strengthening. The coexistence of low serum T3 and muscle enlargement demonstrates the anabolic effect of testosterone. Therefore, T3 and T4 profiles depicted the clamshell and fishbone effect on metabolism in Bangkok roosters.

High levels of T3 and T4 can coincide with thicker pectoral muscle but less abdominal fat in the broiler [8, 11]. Broiler has a thicker intramuscular fat than indigenous Thai chickens [16, 37]. T3 and T4 stimulate fat and protein degradation to increase blood glucose and heat production [9]. High body fat in broiler may replace the protein degradation to sustain protein synthesis and muscle growth. The favor of β-hydroxy-β-methyl butyrate calcium (HMB-Ca) can support muscle growth and protein synthesis in the broiler with high thyroid hormones [11]. Dietary HMB-Ca is a leucine metabolite that potentially promotes muscle mass in animals and humans [11]. The Bangkok rooster is originally an indigenous game bird in Thailand [13, 14]. Cockfighting roosters typically develop huge muscles and aggressive behavior [12]. The decline of T3 in P1 could assist muscle enlargement by slowing down protein catabolism. The scarcity of fat predisposes skeletal muscle to have T3-induced protein degradation in Bangkok rooster. Excess T3 activity causes excessive gluconeogenesis in the musculus that proceeds protein degradation [33]. The low T3 but high T4 activity also indicates a healthy metabolism that supports muscle growth in Bangkok roosters.

5 Conclusion

Clamshell and fishbone treatments reduce T3 but increase T4 activity in Bangkok roosters. Clamshells and fishbones also stimulate fasciculus enlargement. This study suggested that the clamshell and fishbone combination was an effective muscle promotor for chicken. Clamshell and fishbone are potent feed supplements in poultry. The discovery of an efficacious feed supplement can contribute to sustainable and precision husbandry.

Acknowledgments. This research was supported by Universitas Gadjah Mada with Batch I Rekognisi Tugas Akhir (RTA) Grant 2022 with certificate number 3550/UN1.P.III/Dit-Lit/PT.0105/2022.

Authors' Contributions. All authors confirm the following contributions to the paper: research conception and design: Pudji Astuti; data collection: Sarmin Sarmin; analysis and interpretation of results: Claude Mona Airin; draft manuscript preparation: Akhmad Abror As Sidiqi. The final manuscript had approval from all authors.

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