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Comparison of several techniques to determine the lipid profile in "muscovy duck" *cairina moschata*, Linnaeus, 1758 (Anseriformes: Anatidae)*

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Abstract

The lipid metabolism of domesticated birds has been studied by altering their diet to increase the amount of polyunsaturated fatty acids in the triglycerides and decrease the total cholesterol in the blood and yolk, which is used for human consumption. The Cairina moschata domestica species can be used to produce meat and eggs. The animals were raised at Vereda Morro Gordo, El Remanso Farm, located in the municipality of Manizales (Colombia), at 1800 m above sea level (23 °C). A total of 79 creole ducks (37 females and 42 males) were raised for 6 months and put under a 12 hours-of-light regime. Several methods to determine the lipid profile in this species were examined: two for high-density lipoprotein cholesterol (HDL-C) and three for low-density lipoprotein cholesterol (LDL-C). All the reagents belonged to BioSystems S.A. laboratories, from Barcelona, Spain. Assays were carried out in the RAYTO RT-1904C device, a semiautomatic chemistry analyser. In both cases the direct determination method is recommended; however, TAG levels should not exceed the maximum permitted by manufacturing laboratories. Females showed hypertriglyceridemia when compared to males. There are statistically significant differences as per sex in the values of HDL-cholesterol, LDL-cholesterol, and triglycerides, but not in the total cholesterol values.

Keywords: Lipids, techniques, duck, metabolism.

Comparación de técnicas para determinar el perfil lipídico en "pato real" *cairina moschata*, Linnaeus, 1758 (Anseriformes: Anatidae)

Resumen

El metabolismo lipídico de aves domésticas ha sido objeto de estudio mediante la modificación de su dieta para incrementar la cantidad de ácidos grasos poliinsaturados en los triglicéridos y disminuir el colesterol total en la sangre y la yema, esta última utilizada para consumo humano. La especie Cairina moschata domestica se emplea para la producción de carne y huevos. Los animales fueron criados en la Vereda Morro Gordo, Finca El Remanso, ubicada en el municipio de Manizales (Colombia), a una altitud de 1800 metros sobre el nivel del mar y a una temperatura de 23 °C. Un total de 79 patos criollos (37 hembras y 42 machos) fueron

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criados durante 6 meses bajo un régimen de 12 horas de luz. Se examinaron varios métodos para determinar el perfil lipídico en esta especie: dos para el colesterol de lipoproteínas de alta densidad (HDL-C) y tres para el colesterol de lipoproteínas de baja densidad (LDL-C). Todos los reactivos pertenecían a los laboratorios BioSystems S.A., con sede en Barcelona, España. Los ensayos se llevaron a cabo en el dispositivo RAYTO RT-1904C, un analizador químico semiautomático. En ambos casos se recomienda el método de determinación directa; sin embargo, los niveles de TAG no deben exceder el máximo permitido por los laboratorios fabricantes. Las hembras mostraron hipertrigliceridemia en comparación con los machos. Se observaron diferencias estadísticamente significativas según el sexo en los valores de colesterol HDL, colesterol LDL y triglicéridos, pero no en los valores de colesterol total.

Palabras clave: Lípidos, técnicas, pato, metabolismo.

Introduction

The creole duck (Cairina moschata domestica) is a duck species of the Anatidae family from tropical America and whose distribution area covers from Mexico to Argentina and central Uruguay, in tropical and subtropical climates. There are two varieties that show marked phenotypic differences: the wild and the domesticated. Since pre-Columbian times indigenous communities raised wild ducks from which the domesticated species derived, known in Hispanic America as "creole duck" (Cairina moschata domestica) (Da Silva Costa., 2023), showing important differences caused by selective breeding and a small need to fly for food. Due to its disease resistance and ability to seek food in open spaces with water and vegetation, this duck is suitable for bioproduction systems, where it can be used in other production areas such as fish farms, swine production, fruit farming, and others (Pervin et al., 2013). The lipid metabolism of domesticated birds has been studied by altering their diet to increase the amount of polyunsaturated fatty acids in the triglycerides (TG) and decrease the total cholesterol (TC) in the blood and yolk (Ayerza and Coates, 2000; Chowdhury et al., 2002), which is used for human consumption. TG and TC are transported in the blood to the birds' oocytes through very-low-density lipoproteins (VLDL) containing apolipoprotein VLDL-II (Salvante et al., 2007) and through high density lipoproteins (HLD), also containing apolipoprotein (Eftekhar et al., 2015). Several methods to measure HDL cholesterol (HDL-C) are available. One of them is the precipitation method, consisting of the precipitation of non-HDL lipoproteins by the action of different reagents (which vary according to the manufacturer), proceeding to the determination of HDL-C in the supernatant. Another one is the direct method—also known as homogeneous method—which is specific and free of endogenous interferences such as high blood TG levels (Ueda et al., 2003; Warnick et al., 2001). Current research have studied the lipid metabolism in birds using precipitation techniques on phosphotungstate for HDL C quantification (Salma

et al., 2007; Yin et al., 2008). The ultracentrifugation method, along with an enzymatic method for determining cholesterol levels of said lipoprotein, have also been used (An et al., 2015), as well as the precipitation or the direct method for measuring HDL-C in the blood of laying hens (Liu et al., 2010; Yue et al., 2011). Nevertheless, the feasibility of the available methods to measure the lipid profile of other meat and egg production species such as Cairina moschata must be established, which is the objective of this research that also provides the lipid profile reference values of this species.

Materials and methods

The animals were raised at Vereda Morro Gordo, El Remanso Farm, located in the municipality of Manizales (Colombia), at 1800 m above sea level, with an average temperature of 23 °C. The laboratory determinations were carried out in the Metabolism Laboratory of the Universidad de Manizales. The total population of the study is 441 ducks, and the sample was calculated using finite population formulas with a maximum estimation error of 10% (Aguilar, 2005). This resulted in a sample of 79 creole ducks (37 females and 42 males) were raised for 6 months and put under a 12 hours-of-light regime. A laying-hen commercial standard diet was administered. Water was provided ad libitum. Before the blood drawing, the birds were fasted during 12 hours. 5 ml of blood were directly extracted from the jugular vein in test tubes. The blood was separated in a Hettich zentrifugen EBA 20 (Tuttlingen, Germany) centrifuge at 3000 revolutions per minute (rpm) for 5 minutes at room temperature. The serum obtained was frozen at -30 °C until its subsequent assay. At the moment of processing the samples, the sera were thawed at room temperature and put in bain-marie at 37 degrees C for 10 minutes. All the reagents belonged to BioSystems S.A. laboratories, from Barcelona, Spain. The total cholesterol (TC), triglycerides (TAG), HDL cholesterol (HDL-C), and LDL cholesterol (LDL-C) values were determined by enzymatic colorimetric methods following the guidelines of the manufacturer. Assays were carried out in the RAYTO RT-1904C device, a semiautomatic chemistry analyser.

Determination of HDL cholesterol using the direct method (detergent)

This method consists of: A Reagent A containing a Good buffer, <1 U/ml of cholesterol oxidase, <1 U/ml of peroxidase, 1 mmol/L of N.Nbis (4-sulfobutyl)-m-toluidine, and 1 mmol/L of an accelerant. A Reagent B comprising a Good buffer, <1.5 U/ml of cholesterol esterase, 1 mmol/L of 4 AA, <3.0 KU/L of ascorbate oxidase, and a detergent. 7 µl of the serum sample were taken and 750 µl of Reagent A were added. Here, the cholesterol of portomicrons (PM) (chylomicrons in mammals), VLDL, intermediate-density lipoproteins (IDL), and low-density lipoproteins (LDL) were hydrolysed by cholesterol esterase (CO)

in a non-colour-forming reaction. Immediately after this, 250 µl of Reagent B were added to the product of the previous step. The mixture was incubated at 37 °C for 5 minutes. HDL cholesterol was dissolved using a detergent; cholesterol esterase and cholesterol oxidase hydrolysed HDL cholesterol, and cholestenone and hydrogen peroxide were formed as end products. Hydrogen peroxide, along with DSBmT and 4- aminoantipyrine (4-AA) reacted with peroxidase, generating quinoneimine and four water molecules. Quinoneimine is proportional to the concentration of HDL cholesterol in the sample, which was quantified spectrophotometrically.

Determination of HDL cholesterol using the precipitation method (phosphotungstate)

For this method, 1 ml of reagent (phosphotungstate 0.4 mmol/L and magnesium chloride 20 mmol/L) was used and mixed with 0.2 ml of the serum sample. It was thoroughly stirred and left at room temperature for 10 minutes and centrifuged at 4000 rpm for 10 minutes. VLDL, IDL, and LDL remained in the precipitate and HDL in the supernatant. Finally, 100 µl of the supernatant were transferred, deposited in other test tube, mixed with 1 ml of the reagent for TC (described in the section below), and incubated for 10 minutes in bain-marie at 37 °C. Cholesterol esterase and cholesterol oxidase hydrolysed HDL-C, causing a hydrogen peroxide formation that was consumed by peroxidase in the presence of 4-AA and phenol. This caused quinoneimine as end product, proportional to the HDL-C sample, which was quantified spectrophotometrically.

Determination of LDL cholesterol using the Friedewald Formula

TC, HDL-C, and TAG levels were determined in serum. TC determination in serum was carried out mixing 10 µL of the sample and 1 mL of the reagent (35 mmol/L of Pipes, 0.5 mmol/L of sodium cholate, 28 mmol/L of phenol, >0.2 U/mL of cholesterol esterase, >0.1 U/mL of cholesterol oxidase, >0.8 U/ mL of peroxidase, 0.5 mmol/L of 4-AA, pH 7.0). The mixture was thoroughly stirred and the tubes were incubated for 10 minutes at room temperature. Cholesterol esterase hydrolysed cholesterol esters and originated free cholesterol, which due to the cholesterol oxidase, formed cholestenone + hydrogen peroxide. When coupled with 4-AA and phenol quinoneimine appears by peroxidase action. Quinoneimine is proportional to the total cholesterol of the samples and was quantified spectrophotometrically. The determination of HDL-C was carried out using the precipitation method, as explained in the previous section. The determination of TAG in serum was carried out using 10 µL of the sample and 1 mL of the reagent (45 mmol/L of Pipes, 6 mmol/L of 4-clorophenol, 5 mmol/L of magnesium chloride, >100 U/mL of lipase, >1.5 U/mL of glycerol kinase, >4 U/mL of glycerol-3P-oxidase, >0.8 U/mL of

peroxidase, 0.75 mmol/L 4-AA, 0.9 mmol/L of ATP, pH 7.0). The mixture was thoroughly stirred and the tubes were incubated for 15 minutes at room temperature. In the previous process, triglycerides were hydrolysed by lipase-to-glycerol and fatty acids. In the presence of ATP, glycerol was phosphorylated by glycerol kinase and formed glycerol 3P + ADP. In the presence of oxygen, glycerol 3 formed hydrogen peroxide by the glycerol-3P-oxidase action. Finally, the quinoneimine was quantified spectrophotometrically by the peroxidase action over hydrogen peroxide in the presence of 4-AA and chlorophenol; quinoneimine is proportional to the TAG concentration. LDL cholesterol values were calculated using the following formula: LDL cholesterol = total cholesterol HDL cholesterol VLDL cholesterol. VLDL-C was calculated by dividing triglycerides in 5 (TAG/5) (Tsigalou et al., 2021).

Determination of LDL cholesterol using the direct method (detergent)

This method consists of a Reagent A: >30 mmol/L of MES buffer, <1.5 U/mL of cholesterol esterase, <1.5 U/mL of cholesterol oxidase, 0.5 mmol/L of 4-AA, <3.0 U/L of ascorbate oxidase, >1 U/mL of peroxidase, detergent, pH 6.3, and a Reagent B: >30 mmol/L of MES buffer, 1 mmol/L of DSBmT, detergent, pH 6.3. 750 µL of reagent A and 7 µL of the serum sample were pipetted. The detergent of reagent A dissolved HDL and VLDL cholesterol and portomicrons. Cholesterol esters were hydrolysed simultaneously by cholesterol esterase and cholesterol oxidase, forming cholestenone and hydrogen peroxide. The latter was consumed by peroxidase in the presence of 4-AA. This reaction did not form any colour. 250 µL of reagent B were added to the product of the previous step and incubated for 5 minutes at 37 degrees C. The detergent of said reagent dissolved LDL cholesterol as in the previous step. Cholesterol esterase and cholesterol oxidase hydrolysed LDL cholesterol and formed cholestenone and hydrogen peroxide as end products. The presence of peroxidase condensed hydrogen peroxide and DSBmT to form quinoneimine. Quinoneimine is proportional to the concentration of LDL cholesterol in the sample, which was quantified spectrophotometrically.

Determination of LDL cholesterol using the precipitation method (polyvinyl sulphate)

0.2 ml of the reagent (3 g/L of polyvinyl sulphate, 3 g/L of polyethylene glycol) and 0.4 mL of the serum sample were used. Both were mixed, stirred, and left standing for 15 minutes at room temperature, and then centrifuged at 4000 rpm for 15 minutes. In the procedure, LDLs in the sample were precipitated in presence of polyvinyl sulphate and polyethylene glycol. The latter served as accelerant of the precipitation. VLDL, IDL, and HDL remained in the supernatant. Finally, 20 µl of the supernatant were carefully collected. The supernatant was deposited in other test tube, mixed with 1 ml of the reagent for TC and incubated for

10 minutes in bain-marie at 37 °C. The supernatant cholesterol was quantified spectrophotometrically by the following coupled reactions: cholesterol esterase and cholesterol oxidase formed cholestenone and hydrogen peroxide; the action of peroxidase and 4-AA formed quinoneimine. This is proportional to VLDL, IDL, and HDL cholesterol. The LDL cholesterol concentration was calculated using the following formula: LDL-C = TC - the supernatant cholesterol.

Statistical analysis

ANOVA was used for the analysis of data and IBM SPSS Statistics for the statistical analysis. Statistically significant differences were determined with $P < 0.05$ and the F-test. The Pearson correlation coefficient was evaluated between TC, TAG, HDL-C, VLDL-C, and LDL-C, with a $P < 0.05$ significance.

Ethical considerations

For this study encompassed obtaining approval from the University of Manizales, in accordance with established research and animal welfare guidelines.

Results

The results showed significant differences between males and females in the parameters analysed, except for total cholesterol (Table 1). The mean of TAG, HDL-C, LDL-C, and VLDL-C showed the following values (mg/dL) for males: 73.8, 27.3, 116, and 14.8 respectively, whereas in females, the values (mg/dL) were: 300.5, 22.3, 89.1, and 60.1 respectively, indicating higher levels in females. Hypertriglyceridemia was also observed in females. The P-value of the F-test, below 0.05, showed a significant difference as per sex for said values, with a 95% confidence level. The total cholesterol value indicated 173.6 mg/dL for males, whereas in females the value was 181.5 mg/dL, meaning they were higher in females. The P-value of the F-test, over 0.05, showed that said values have no statistically significant differences as per sex, with a 95% confidence level.

Tabla 1. Concentrations of cholesterol, triacylglicerydes, HDL-C, VLDL-C, and LDL-C in Cairina moschata.

Parameter	Males			Females			P-value
	Mean	Statistics		Mean±SD	Statistics		
Triglycerides	73.8±24	Minimum	25.8	300.5±265.2	Minimum	65.8	0.000
		Maximum	142.1		Maximum	1016	
Total cholesterol	173.6±42.3	Minimum	102.7	181.5±48.7	Minimum	65	0.444
		Maximum	310.6		Maximum	294.3	
HDL-C direct	27.3±7.4	Minimum	16.2	22.3±8.8	Minimum	9.4	0.007
		Maximum	46.2		Maximum	42	
HDL-C precipitation	26.5±7.5	Minimum	14.3	21.1±8.9	Minimum	8.4	0.005
		Maximum	44.6		Maximum	42.1	
LDL-C direct	116±37	Minimum	61.9	89.1±48.6	Minimum	14.6	0.007
		Maximum	259.1		Maximum	192.4	
LDL-C precipitation	114.8±36.5	Minimum	67.2	84.9±48.7	Minimum	20.9	0.003
		Maximum	262		Maximum	190.5	
LDL-C formula	131.5±39.6	Minimum	64.5	99.1±54.7	Minimum	12.5	0.003
		Maximum	268.8		Maximum	221	
VLDL-C formula	14.8±4.8	Minimum	5.2	60.1±53	Minimum	13.2	0.000
		Maximum	28.4		Maximum	203.2	

Statistically significant differences were found by comparing methods for determining HDL cholesterol; the direct method based on a detergent + DSBmT is reliable, given it is appropriate for serum samples with high levels of triglycerides (up to 3000 mg/dL). No statistically significant difference was found between the direct and precipitation methods to determine LDL cholesterol. A significant difference was found by comparing the formula method vs the direct method and the precipitation method (Table 2).

Table 2. Comparison of the direct and precipitation methods and the LDL-cholesterol determination formula, and the precipitation and direct methods for the determination of HDL-C.

Comparison of methods				
No.	**Measuring method**	**Mean (mg/dL)**	**DS**	**P-value**
1	HDL-C direct	25.0	8.4	.000
	HDL-C precipitation	24.0	8.6	
2	LDL-C direct	103.4	44.6	.076
	LDL-C precipitation	100.8	45.0	
3	LDL-C formula	116.3	49.7	.000
	LDL-C precipitation	100.8	45.0	
4	LDL-C formula	116.3	49.7	.000
	LDL-C direct	103.4	44.6	

Discussion

When lipid metabolism research is conducted to study different animal species, it is important to consider that cholesterol assay methods for lipoproteins are analysed or used in healthy humans, usually in fasting conditions (Ueda et al., 2003) or suffering from metabolic diseases, such as hypertriglyceridemia (Osorio & Flórez, 2014) or diabetes (Miida et al., 2017; Guerra et al., 2005), among others. In the case of laying hens, different lipid metabolism research have been conducted with the available methodology, giving specific HDL-C values and their low or high impact on the yolk, but without considering if the data obtained corresponds to the reality of the animal, since high blood TAG levels in females might alter the results of the methods used. The higher triglyceride (TAG) content in female muscovy ducks compared to males suggests a specific metabolic adaptation related to the energetic demands of reproduction. This condition is relevant for females as it provides an additional energy reserve to efficiently support the reproductive process, including egg production and incubation, thereby potentially enhancing their ability to sustain and rear offspring (Lin et al., 2022). In this research, high-density lipoproteins cholesterol was analysed, showing that serum levels vary significantly in the two methods analysed (Table 2).

It is important to bear in mind that the analysis of the precipitation method on phosphotungstic + Mg, consists of forming a complex of apolipoproteins B (apo B) of VLDL, IDL, and LDL with the divalent polyanions and cations containing the reagents (Hafiane & Genest, 2015), causing said lipoproteins to sediment. However, this procedure is altered when triglycerides levels are above 400 mg/dL in serum (Rajagopal et al., 2012),

forming a complex that prevents the entire precipitation of non-HDL lipoproteins, resulting in HDL-C concentrations that exceed the actual values (Hosseinzadeh et al., 2020). Furthermore, most female samples showed hypertriglyceridemia. The direct method to quantify HDL-C, besides reducing the operational costs, has the advantage of suppressing the reaction of PM, VLDL, ILD, and LDL cholesterol and measures HDL-C directly, without being affected by the high levels of TG in serum (Warnick et al., 2001). Moreover, Biosystems laboratories indicate that TG values of 3000 mg/dL are acceptable, demonstrating that using this method in laying hens is safer. To measure LDL-C with the Friedwald formula, concentrations of TC, HDL-C, and TAG in serum were considered, and VLDL-C was calculated using the TAG/5 formula. Several research studies demonstrated that this formula worked in healthy humans with TAG levels below 400 mg/dL, which had a 5:1 ratio between TAG, TC, and the VLDL (Tsigalou et al., 2021). This relationship changes in females, given the TC proportion is lower and TAG in the VLDL proportion is higher (Hermier, 1997). Therefore, the Friedewald formula is not feasible for Cairina moschata or for the calculation of VLDL-C, and let alone for the calculation of LDL-C, due to the amount of errors in the equations needed to obtain the required results. Liquid homogeneous assay techniques, also known as direct methods for measuring LDL C, have been said to be free of problems to patients without fasting conditions, and that they can measure LCL-C in samples in which TAG levels were above 400 mg/dL (Fei et al., 2000). However, the method used in this research (detergent + DSBmT) has negatively altered the results, as high TAG concentrations in serum exceed the maximum range permitted (Islam et al., 2022); the reagents used in this research, belonging to BioSystems laboratories, accept a lipaemia with a TAG maximum of 1290 mg/Dl. The Friedewald method might cause errors in the results given the three sample measurement processes, which becomes more evident when the species analysed has high levels of triglycerides above 400 mg/dL (Tsigalou et al., 2021). This situation occurs in laying hens (Salma et al., 2007) and in the Cairina moschata females of this research. Nevertheless, that does not occur in broilers—since hypertriglyceridemia is not observed (Hermier, 1997; Musa et al., 2007; Velasco et al., 2010)—or in Cairina moschata males.

Finally, the comparison of techniques for determining the lipid profile in "muscovy duck" cairina moschata is essential for ensuring the accuracy of analytical methods in this species, as indicated by the findings of the present study. This leads to increased precision and optimization when selecting an appropriate technique. By contrasting different techniques, potential discrepancies in results can be identified and the precision and accuracy of each method evaluated. Furthermore, this process enables the selection of the most suitable techniques for the specific needs of each study or application, ensuring the reliability of the obtained data. Comparing techniques also contributes to the development of quality standards and analysis protocols for lipid profile assessment in Muscovy ducks, thereby enhancing the consistency and reproducibility of results across various research environments and contexts (Arias-Sosa & Rojas,b 2021)

Conclusion

The measurement of LDL-C using the Friedewald formula and the measurement of HDL-C levels using the precipitation method should not be used for Cairina moschata, since high TAG levels in serum will have a negative impact on the cholesterol values of these lipoproteins. The direct method was found to be feasible in this species; however, TAG levels should not exceed the maximum permitted by manufacturing laboratories.

Declaration of conflict of interest

The author states there are no economic conflicts of interest, or of any other kind, that may indicate this research has been biased in any way.

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