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Gallus gallus domesticus (Galliformes: Phasianidae) as a biological model for biosafety analysis of the hexane fraction of the fruits and seeds of *Ricinus communis* L. (Malpighiales: Euphorbiaciae)

Gallus gallus domesticus (Galliformes: Phasianidae) como modelo biológico para análise de biossegurança da fração hexânica de frutos e sementes de *Ricinus communis* L. (Malpighiales: Euphorbiaciae)

Juliana Marceli Hofma Lopes[1]; Patricia Franchi de Freitas[2]; Everton Ricardi Lozano[3]; Edgar de Souza Vismara[4]; Elizabete Artus Berté[5]; Silvane Zancanaro de Oliveira[6]; Camila Maria Zankanol Griebeler[6]; Michele Potrich[7]*

Highlights

The botanical insecticide from *Ricinus communis* is toxic to several target organisms.
This insecticide caused toxicity to *Gallus gallus domesticus* embryos.
Ricinus communis caused malformations in the embryos of *Gallus gallus domesticus.*

Abstract

Brazil became the largest consumer of pesticides in the world in 2021. These products, although necessary, can cause significant damage to non-target organisms and agroecosystems. From this perspective, the search for new, safer products gains prominence every year. It is essential to understand

[1] Doctoral Student of the Postgraduate Program in Biotechnology, Universidade Tecnológica Federal do Paraná, UTFPR-DV, Campus Dois Vizinhos, Ponta Grossa, Brazil. E-mail: julianamarceli@alunos.utfpr.edu.br
[2] Profª Drª, Coordination of Biological Sciences, UTFPR-DV, Campus Dois Vizinhos, Brazil. E-mail: patriciafreitas@utfpr.edu.br
[3] Prof., Postgraduate Program in Agroecosystems, UTFPR-DV, Campus Dois Vizinhos, Brazil. E-mail: evertonlricardi@utfpr.edu.br
[4] Prof., Postgraduate Program in Animal Science, UTFPR-DV, Campus Dois Vizinhos, Brazil. E-mail: edgarvismara@utfpr.edu.br
[5] Doctoral Student of the Postgraduate Program in Biological Sciences, Universidade Estadual de Londrina, UEL, Brazil. E-mail: elizabeteberte9@gmail.com
[6] Master's Degree in the Postgraduate Program in Agroecosystems, UTFPR-DV, Campus Dois Vizinhos, Brazil. E-mail: silvanezanoli@gmail.com; camilagriebeler@alunos.utfpr.edu.br
[7] Director of Research and Postgraduate Studies, DIRPPG, UTFPR-DV, Campus Dois Vizinhos, Brazil. E-mail: michelepotrich@utfpr.edu.br
* Author for correspondence

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how non-target organisms react to contact with these novel products to verify their safety. Therefore, vertebrate models, such as the embryo of *Gallus gallus domesticus* L., 1758 (Galliformes: Phasianidae), an organism from which the results can be extrapolated to other vertebrates, are important to guarantee the selectivity and safety of new products. The objective of this study was to evaluate the effect of the hexane fraction of fruits and seeds of *Ricinus communis* (HFFSRc) on *Gallus gallus domesticus* (the domestic chicken). This fraction has been studied as a potential insecticide for agricultural use. Three concentrations of HFFSRc were evaluated, 1%, 1.5%, and 2%, in addition to two control treatments, one with distilled water and one with Tween 80® (0.01%). The effect of HFFSRc on *G. gallus domesticus* embryos was analyzed through two different forms of exposure: i) injection of treatments into the egg's air chamber and ii) spraying of treatments on the egg. After three days of incubation, the eggs of *G. gallus domesticus* were collected for morphological analysis using the total mount technique. It was observed that their exposure to HFFSRc, regardless of the concentration or form of exposure used, reduced the survival probability of *G. gallus domesticus* embryos. HFFSRc was found to be toxic to *G. gallus domesticus* embryos when sprayed on the eggshell, reducing their survival probability. HFFSRc was also toxic when injected into the air chamber, in addition to causing body malformations in *G. gallus domesticus* embryos.
Key words: Ecotoxicology. Botanical insecticide. Castor bean. Toxicity.

Resumo

O Brasil se tornou o maior consumidor de agrotóxicos do mundo em 2021. Esses produtos, embora necessários, podem causar danos significativos a organismos não-alvo e aos agroecossistemas. Nessa perspectiva, a busca por novos produtos mais seguros ganha destaque a cada ano. Para verificar a sua segurança, é essencial compreender como os organismos não-alvo reagem ao contacto com eles. Portanto, modelos de vertebrados, como o embrião de *Gallus gallus domesticus* L., 1758 (Galliformes: Phasianidae) (organismo que permite extrapolar os resultados para vertebrados) são importantes para garantir a seletividade e segurança de novos produtos. Objetivou-se avaliar o efeito da fração hexânica de frutos e sementes de *Ricinus communis* (HFFSRc) sobre *G. gallus domesticus* (galinha doméstica). Esta reação tem sido estutada como um potencial insetida para uso agrícola. Foram avaliadas três concentrações da HFFSRc, sendo elas, 1%, 1,5% e 2%, além de dois tratamentos controles, sendo um controle com água destilada e um controle com Tween 80® (0,01%). A HFFSRc foi analisada sobre embriões de *G. gallus domesticus* em duas formas diferentes de exposição: i) injeção dos tratamentos na câmara de ar do ovo e ii) pulverização dos tratamentos sobre o ovo. Os ovos de *G. gallus domesticus,* após os três dias de incubação, foram coletados para análise morfológica através da técnica de montagem total. Após a análise, observou-se que a exposição destes a HFFSRc, independentemente da concentração ou forma de exposição utilizada, causou redução na probabilidade de sobrevivência de *G. gallus domesticus,* bem como, a HFFSRc causou malformações nos embriões de *G. gallus domesticus* quando injetada na câmara de ar. Verificou-se que a HFFSRc é tóxica para embriões de *G. gallus domesticus,* reduzindo sua probabilidade de sobrevivência quando pulverizada na casca do ovo, esta HFFSRc quando injetado na câmara de ar também foi toxica além de causar malformações corporais nos embriões de *G. gallus domesticus.*
Palavras-chave: Ecotoxicologia. Inseticida botânico. Mamona. Toxicidade.

Introduction

Agricultural expansion in the last century has raised concerns about the presence of pests in crops. This expansion, consequently, has also increased the amount of pesticides used to control these harmful organisms (Martinez et al., 2017). However, various factors, such as the form of application or the frequency of use, make pesticides a risk to human health, often resulting in genetic and physical damage (Marcelino et al., 2019).

The excessive use of synthetic pesticides can be avoided by employing alternative methods to control insect pests. Among these methods, botanical insecticides are gaining increasing prominence as they can have high selectivity and low toxicity to non-target organisms present in agroecosystems (Dantas et al., 2019). Botanical insecticides originate from a plant's secondary metabolism (Wiesbrook, 2004; Borges & Amorim, 2020). These metabolites act in the plant's defense as repellents and inhibitors of insect feeding (Saito et al., 2004; Santana et al., 2022).

Castor bean plant extract, a botanical insecticide, has been studied for such use (Cunha, 2006). The secondary metabolites of the castor bean plant *Ricinus communis* L. (Malpighiales: Euphorbiaceae) include flavonoid compounds (Sun et al., 2011; Zhang et al., 2011), sterols (Sun et al., 2011), terpenes (Péres et al., 2006), tannins, and total phenolics (Aspé & Fernández, 2011; Yeboah et al., 2021), which can inhibit the development and biological cycle of insects or even kill them, making this extract an alternative for insect control (Bordin et al., 2023).

The hexane fraction from the fruits and seeds of *R. communis* has already proven effective in controlling insect pests such as the soybean looper *Chrysodeixis includens* (Walker, [1858]) (Warmling, 2018) and caterpillars from the Spodoptera complex (Bordin et al., 2023). This fraction contains five compounds identified in the HFFSRC: three flavonoids, one ricinoleic acid, and one cinnamic acid (Table 1). However, this fraction lacks selectivity and may have a negative impact on non-target organisms, such as *Trichogramma pretiosum* Riley, 1879 (Allein et al., 2024). It is important to gather more information about this botanical insecticide's lethal and sublethal effects on non-target organisms. Since biosafety is a key factor in deciding whether to implement new botanical insecticides, testing these products on a vertebrate biological model is essential (Marangoni et al., 2012).

Table 1
Chemical Characterization of the Hexane Fraction of the Fruits and Seeds of Ricinus communis by UHPLC-ESI-QTOF-MS/MS in Negative Mode

			m/z				
No.	Compound	Retention Time (min)	Molecular Formula	Measured Mass (Da)	Theoretical Mass (Da)	Ion Fragments	Class
1	Naringenina	42.6	$C_{15}H_{12}O_5$	271.0552	271.0610	150.9997 (100) 119.0456 (36)	Flavone
2	"Prunin 3"-p-coumarate	44.3	$C_{30}H_{28}O_{12}$	579.1356	579.1580	271.0550 (100) 307.0772 (35) 145.0258 (46)	Flavone
3	Ricinoleic acid	48.3	$C_{18}H_{34}O_3$	297.2375	297.2435	183.1350 (72) 279.2271 (8)	Fatty acid
4	Melilotoside	49.2	$C_{15}H_{18}O_8$	325.1765	325.1840	183.0071 (46) 119.0485 (19)	Cinnamic acid
5	5,6,2'-Trimethoxyfl avone	50.2	$C_{18}H_{16}O_5$	311.1626	311.1685	183.0070 (36) 119.0484 (16)	Flavone

Source: Bordin et al. (2023).

Among biological models, the bird embryo stands out in research related to early embryonic development since its first stages are similar to those of all vertebrates, especially during organogenesis (Irie & Kuratani, 2011). Bird embryos are prominent in comparative embryology due to some of their favorable attributes, such as bird eggs being of the Telolecite type, allowing their complete development in the laboratory; their short total development time; and the fact that embryos develop without a maternal presence, thus avoiding maternal exposure to various manipulations or even death (Barbosa, 1995; Schoenwolf, 1999). Furthermore, the description of the complete embryonic development of *Gallus gallus domesticus* (Galliformes: Phasianidae), the bird most used in laboratory studies, is easily found in the literature (Hamburger & Hamilton, 1951).

Thus, the present work analyzes the biosafety of the botanical insecticide made from the hexane fraction of fruits and seeds of *R. communis* using *G. gallus domesticus* in its initial embryonic development as a biological model.

Material and Methods

This work was carried out at the Biological Control Laboratory (LABCON) of the Federal Technological University of Paraná Campus Dois Vizinhos (UTFPR). This work is registered with the Ethics Committee on the Use of Animals (CEUA/UTFPR) under certificate number 2018-40/2018-CEUA, process 23064.046670/2018-61.

Obtaining HFFSRc and G. gallus domesticus Embryos

HFFSRc was prepared from whole fruits. These were collected in an area of native vegetation. The collected plant material was dried in the shade for approximately 24 h at room temperature (±25°C). It was then placed on Kraft paper (60 x 80 cm) and dried in a forced circulation drying oven for 48 h at 60°C, following Allein (2021).

After the fruits and seeds of *R. communis* were dried and the weight evaluated, grinding was carried out in a Willey knife mill (ALPAX) until the entire vegetable mass was a powder with a particle size of approximately 0.5mm. Then, 20 g of this powder was diluted in 200 mL of 80% alcohol in a 250 mL Erlenmeyer flask. This mixture was placed in a thermostated water bath at 60°C for 30 minutes. Subsequently, the product obtained was filtered in a Kitasato flask with an 8µ filter membrane, UNIFIL brand, with the aid of a vacuum pump (TECNAL TE-058) at a constant pressure of 1.2 kgf cm^2.

After filtering, the product was subjected to the rotary evaporation process in a rotary evaporator (MARCONI MA 120) at a temperature between 55 and 60°C. The evaporator was coupled to a vacuum pump under a constant pressure of 0.35 kgf cm^2 to remove the alcohol. After this, the remaining compounds were resuspended in distilled water until reaching a final volume of 200 mL, forming the crude extract.

The crude extract was fractionated using the liquid-liquid extraction technique (Snyder et al., 1997). A separation funnel with a volumetric capacity of 1,000 mL was used to mix 250 mL of crude *R. communis* extract and 250 mL of the hexane extractor. The mixture was manually stirred for about a minute to obtain greater homogenization, and after 15 minutes of rest, two fractions of this extract were separated. The crude extract was deposited in the lower part of the funnel and the hexane fraction in the upper part, with the part of interest being collected through the tap of the separation funnel. The hexane fraction was again sent to the rotary evaporator (MARCONI MA 120) at a temperature of 42 to 45°C to remove the solvents completely, thus obtaining the hexane fraction of the fruits and seeds of *R. communis* (HFFSRc) used in this work. The HFFSRc was further diluted in 0.01% Tween 80® to obtain standard concentrations of 1%, 1.5%, and 2% for the experiment (Bordin et al., 2023).

Fertilized *G. gallus domesticus* eggs, without cracks and weighing between 60 and 65 g, were used for the experiment. The eggs were purchased from a regional commercial hatchery. Upon arrival at LABCON, they were cleaned with paper towels, moistened with 70% ethyl alcohol, and divided into the treatment groups described in Table 2.

Table 2
Treatments used to evaluate the effect of *Ricinus communis* on survival and morphometry in *Gallus gallus domesticus*

Treatment	Description
Treatment 1 (T1)	1% HFFSRc + 99% 0.01% Tween 80® solution
Treatment 2 (T2)	1.5% HFFSRc + 98.5% 0.01% Tween 80® solution
Treatment 3 (T3)	2% HFFSRc + 98% 0.01% Tween 80® solution
Treatment 4 (T7)	Control closed
Treatment 5 (T8)	0.01% Tween 80® solution

Note. HFFSRc = Hexane fraction of fruits and seeds of *Ricinus communis.*

G. gallus domesticus bioassays and exposure method

The experimental design was divided into nine treatments. Sixty eggs were used for each treatment, totaling 540 eggs for all exposure mediums. The embryos were subjected to two different types of exposure, the first being through injections made directly into the egg's air chamber and the second through spraying of the egg's surface using an airbrush. The concentrations used for each treatment were: T1: 1% of HFFSRc + 99% 0.01% Tween 80® solution; T2: 1.5% of HFFSRc + 98.5% 0.01% Tween 80® solution; T3: 2% of HFFSRc + 98% 0.01% Tween 80® solution; T4: control-closed; and T5: 0.01% Tween 80® solution.

For injection exposure, previously established concentrations were inoculated directly into the air chamber to allow for better dispersion of the agents in the embryo. To inject the selected treatment, a needle (1.60 x 40 mm) was used to pierce the shell, and a standard volume of 100 µL was injected with a syringe and a 0.3 x 8 mm insulin needle (Yamamoto et al., 2012). This is a standard methodology used in *G. gallus domesticus* embryos (Korn & Cramer, 2007; Yamamoto et al., 2012). After injection, the eggs were sealed with adhesive masking tape, thus preventing contact between the eggs' internal contents and the environment. The eggs were then positioned with their air chambers facing upwards in the incubator tray and incubated for three days under a controlled temperature of 38°C and constant humidity and ventilation.

For spray-type exposure, eggs were sprayed with 290 µL (volume calculated based on the area of the plate where the solution was sprayed, in relation to the volume of spray solution per hectare, a standard concentration used to simulate field application) of the selected treatment using a Sagyma® airbrush coupled to a Tecnal® pump (TE-058) with constant pressure (1.2 kgf cm). This procedure simulated field conditions in which vertebrate eggs may be exposed during spraying based on the amount of *R. communis* syrup used per hectare typically used for pest control. After spraying, the eggs were placed in a horizontal laminar flow chamber to evaporate all liquids from

the solution. Next, the eggs were positioned with their air chambers facing upwards and incubated for three days under a controlled temperature of 38°C and constant humidity and ventilation.

Feasibility analysis

After three days of incubation, each shell was opened with scissors at the top of the air chamber. A small portion of the albumen was removed to visualize the embryo better, and then a rectangular filter paper with a diamond cutout in the central region was fitted over the embryo to keep it distended.

Embryos were first classified as viable or non-viable. Viable embryos showed embryonic development, even if these embryos later died, while non-viable embryos had none.

Survival analysis

In this analysis, embryos were classified as alive or dead. Embryos were considered alive if they had a heartbeat and dead if they lacked one or had blood clots. The embryos considered alive were counted, and heartbeat analysis was performed. Dead embryos were counted for mortality analysis and subsequently discarded.

Heartbeat analysis

The hearts of live embryos were identified, and the heartbeats counted for 15 seconds. This number was multiplied by four to obtain the number of beats per minute.

Analysis of embryonic development and morphology

Three days after incubation, live *G. gallus domesticus* embryos were euthanized by freezing, following União (2013) standards. This procedure was performed to analyze embryonic development and morphology using the total assembly technique (Yamamoto et al., 2012). For this technique, Carnoy's fixative solution was dripped on the embryo, which, with the aid of tweezers and scissors, was removed from the egg and transferred to a Petri dish containing saline solution, where it was washed and the excess yolk removed. Afterwards, each embryo was transferred from the Petri dish to a Ksete labeled with the data of the embryo in question.

The embryos were then fixed in Carnoy's solution for 2 hours at room temperature in a closed Petri dish. Afterward, they were washed in distilled water and placed in 70% ethyl alcohol, where they remained for at least 8 hours. The embryos were then hydrated in distilled water for 10 minutes and stained with Carmalúmen de Mayer staining solution for 48 hours. After staining, they were dehydrated in increasing concentrations of ethyl alcohol (70%, 90%, and 95% for 10 minutes each, 100% I and 100% II for 15 minutes each) and cleared with two baths of xylene p. a. ACS (10 minutes each). Synthetic Canada balsam and a coverslip were used to mount the slides.

Different treatments were used to analyze and determine the stages of embryonic development of each specimen. Following Hamburger and Hamilton's (1951) description, the size of the wing and leg buds, in addition to the size and curvature

of the tail and the size of the head in relation to the body, were taken into account. The common embryonic stages after 72 hours of incubation are between 16 and 19, also according to Hamburger and Hamilton (1951).

Slides made with the total embryo assembly technique were used for the morphological analysis of the embryos. These slides were analyzed one by one to check the body structures for embryonic malformations. When relevant, photographic recording was carried out using a stereoscopic microscope (Zeiss, Stemi 305) coupled to a camera (Axiocam ERc 5s) and using ZEN 2.3 LITE software.

Statistical analysis

A generalized linear model (GLM) from the binomial family with a logistic link function was applied for the variables feasibility, survival, and malformation, which have binary outcomes (i.e., occurrence or non-occurrence of the event), following the approach of McCullagh and Nelder (2019). These variables were modeled to capture the probability of occurrence, with the general structure of the model representing the relationship between the log odds of the event and the treatment effects.

A Poisson generalized linear model was used for the variable heartbeat, which consists of count data, as this model was appropriate for modeling the relationship between the mean rate of heartbeats and the treatments. The statistical analysis of these four variables followed three key steps, with the final stage being conditional upon the outcome of the second:

1. Model fitting: Maximum likelihood estimation was used to fit the models.
2. Deviance analysis: A chi-square test was conducted to evaluate model deviance. If significant treatment effects were identified, the analysis proceeded to the next stage.
3. Multiple comparisons test: If treatment effects were detected, Tukey's honestly significant difference (HSD) test was applied to perform pairwise comparisons among treatments, ensuring a robust control for Type I error across multiple comparisons.

The non-parametric Kruskal-Wallis's test was utilized for the ordinal variable developmental stage. If a significant treatment effect was detected, Dunn's test was employed for pairwise comparisons (Corder & Foreman, 2011).

Results

Feasibility analysis

There were no changes in the feasibility of *G. gallus domesticus* embryos subjected to the different treatments. The feasibility rate remained between 90 and 100%. This rate was obtained by counting the number of incubated eggs with normally developed embryos, whether the embryos were alive or dead when the egg was opened. That is, a non-viable egg does not contain embryonic cells, while a viable egg, whether alive or dead, does (Cruz et al., 2021). As shown in Figure 1.A and 1.B, treatments T1, T2, and T3 (spraying or injection of HFFSRc) reduced the survival probability of *G. Gallus domesticus,* differing significantly from T4 and T5 (control groups, injection and spraying).

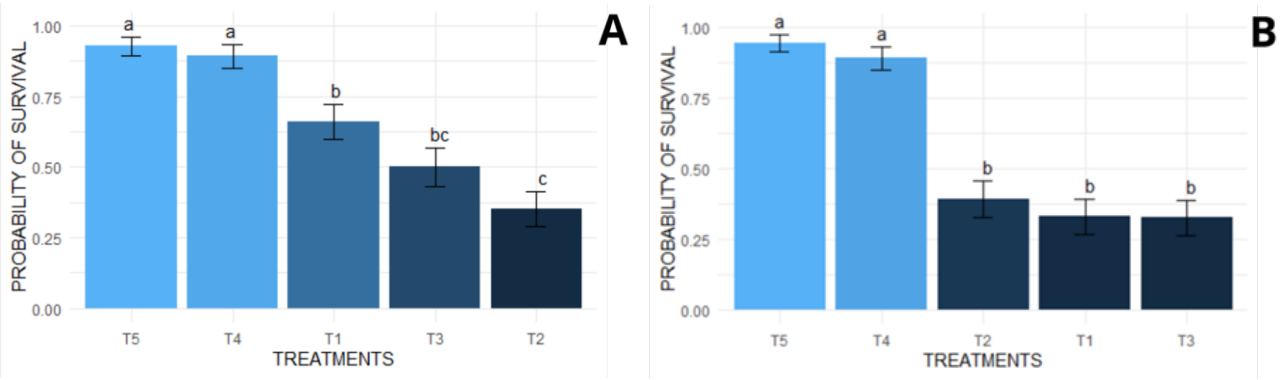

Figure 1. A: Survival Probability of *Gallus gallus domesticus* Embryos Sprayed With Different Concentrations of HFFSRc. B: Survival Probability of *Gallus gallus domesticus* Embryos Injected With Different Concentrations of HFFSRc. T1: 1% HFFSRc; T2: 1.5% HFFSRc; T3: 2% HFFSRc; T4: Control closed; T5: 0.01% Tween®80 solution.

Heartbeat analysis

The average heart rate of G. gallus domesticus embryos subjected to the different treatments was 131. There was a slight reduction in the heart rate of embryos receiving treatment T3 through injection or spraying in relation to those receiving T5 through injection or spraying (Figures 2.A and 2.B). However, no treatment caused a significant reduction when compared to T4, the control closed group.

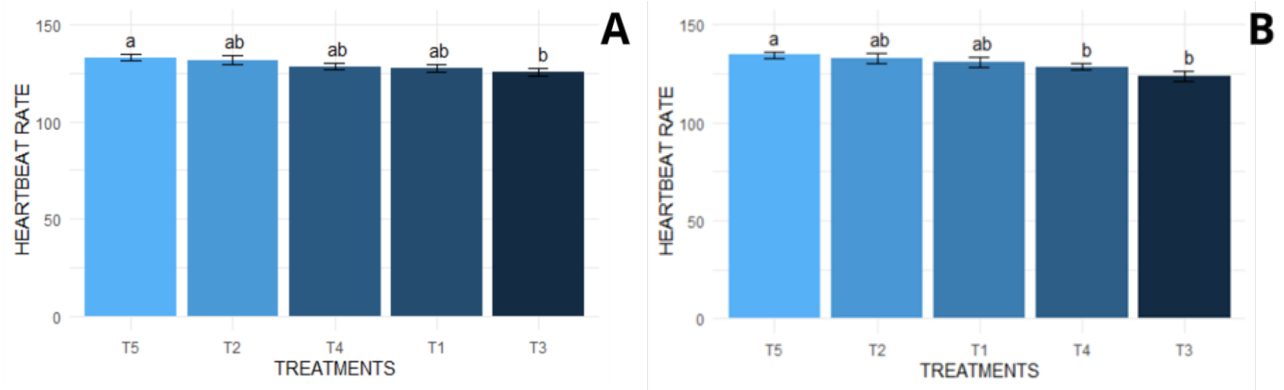

Figure 2. A: Heartbeat Rate of *Gallus gallus domesticus* Embryos Sprayed With Different Concentrations of HFFSRc. B: Heartbeat Rate of *Gallus gallus domesticus* Embryos Injected With Different Concentrations of HFFSRc. T1: 1% HFFSRc; T2: 1.5% HFFSRc; T3: 2% HFFSRc; T4: Control closed; T5: 0.01% Tween®80 solution.

Analysis of embryonic development and morphology

It was observed that in all treatments, the embryos of *G. gallus domesticus* showed the same variation in the stages of development, all being between stages 17 and 20 (Figures 3.A and 3.B), with no changes occurring as a result of the treatments. *G. gallus domesticus* embryos exposed to treatments T1, T2, and T3 by injection had a probability of malformation of between 25 and 50%, differing significantly from the embryos receiving treatments T4 and T5 by injection. Embryos exposed to treatments T1, T2, and T3 by spraying had a probability of malformation of between 10% and 25%. The embryos exposed to control treatments T4 and T5 (by injection or spraying) showed a probability of malformation of below 10%, differing significantly from embryos that received injections of HFFSRc in the air chamber (Figures 4.A and 4.B).

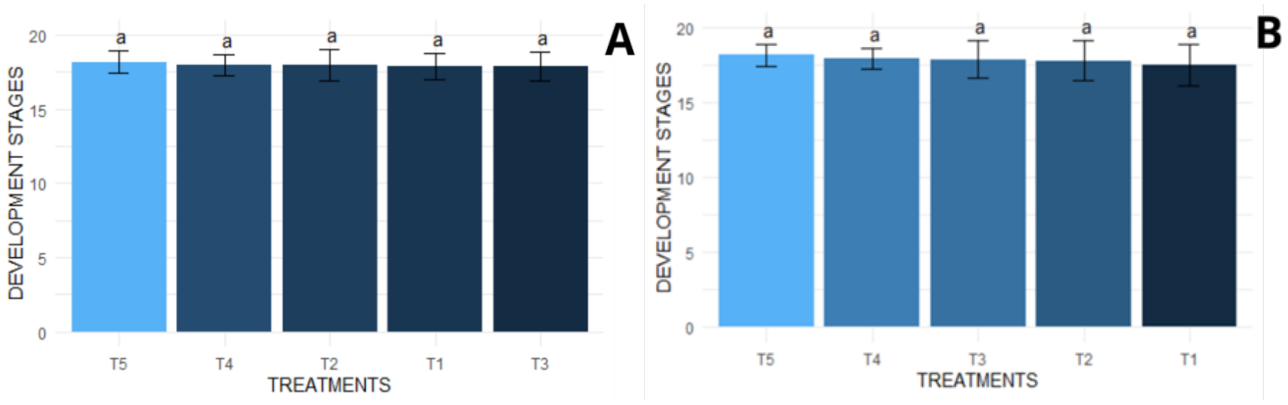

Figure 3. A: Development Stages of *Gallus gallus domesticus* Embryos Sprayed With Different Concentrations of HFFSRc. B: Development Stages of *Gallus gallus domesticus* Embryos Injected With Different Concentrations of HFFSRc. T1: 1% HFFSRc; T2: 1.5% HFFSRc; T3: 2% HFFSRc; T4: Control closed; T5: 0.01% Tween®80 solution.

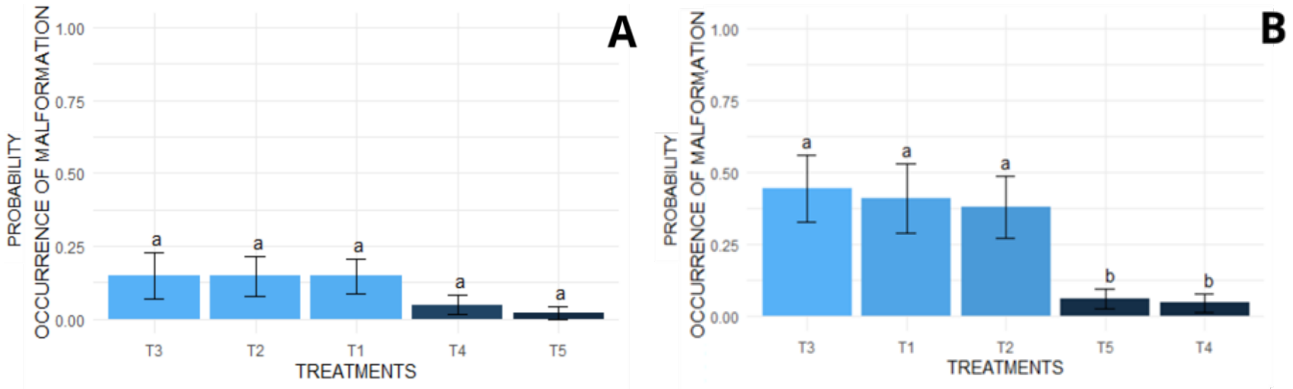

Figure 4. A: Probability of Malformation in *Gallus gallus domesticus* Embryos Sprayed With Different Concentrations of HFFSRc. B: Probability of Malformation in *Gallus gallus domesticus* Embryos Injected With Different Concentrations of HFFSRc. T1: 1% HFFSRc; T2: 1.5% HFFSRc; T3: 2% HFFSRc; T4: Control closed; T5: 0.01% Tween®80 solution.

In all treatments, at least 50% of G. gallus domesticus embryos had intact morphology (Figure 5). The brain region presented only one identified malformation, in which the brain vesicles failed to divide, which was observed in a G. gallus domesticus embryo submitted to T3 treatment (by injection). There were embryos with extensive malformations in all treatments; in most of these embryos, it was impossible to identify their structures or determine their embryonic stage. Additionally, one embryo from the T4 group had a gamma body (two bodies and one head). Extensively malformed embryos are shown in Figure 6.

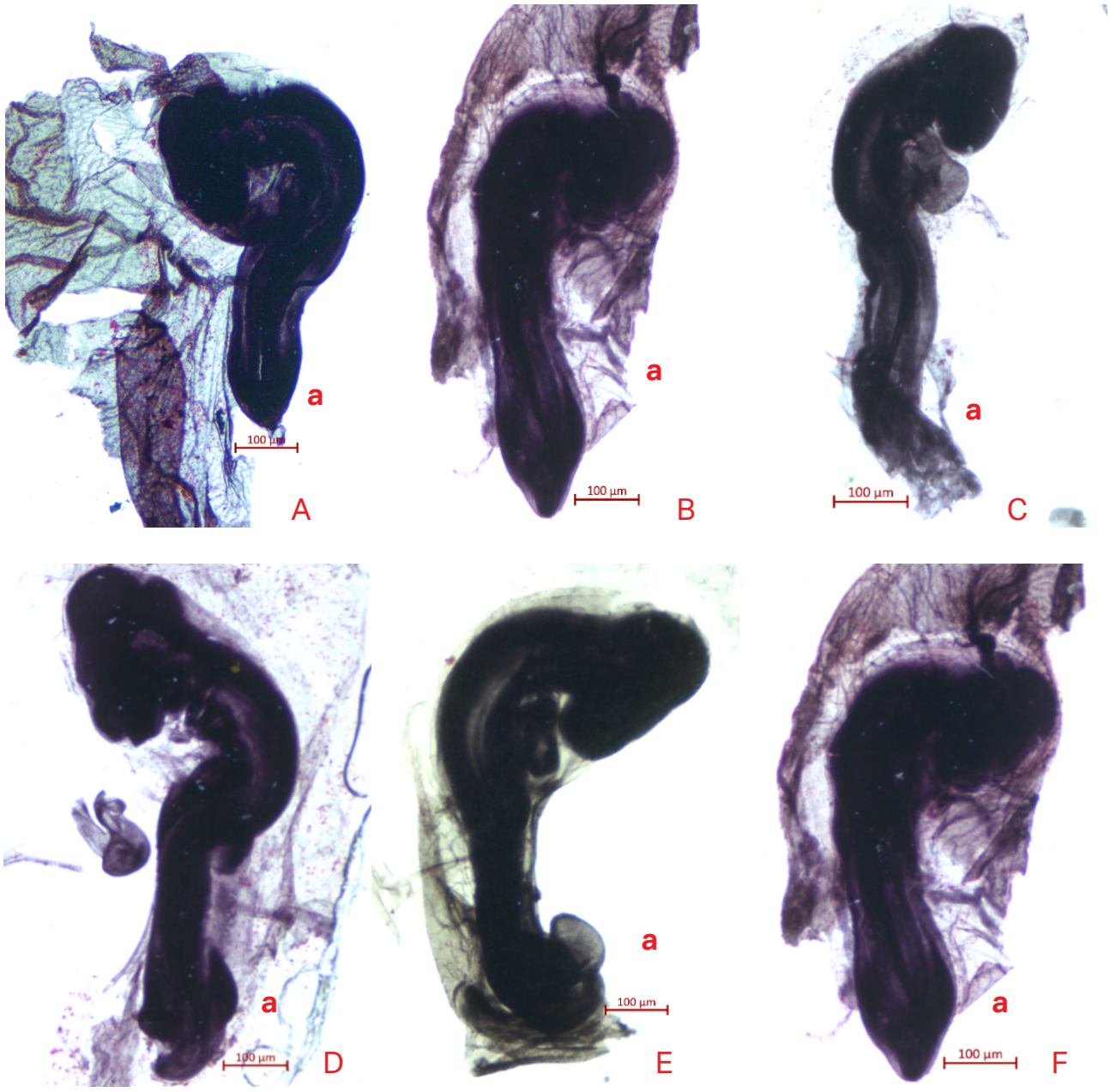

Figure 5. Stereophotography of Gallus gallus domesticus Embryos Considered Normal.
A) T4 Embryo (Closed Control) at Stage 18; B) T5 Embryo (Sprayed With 0.01% Tween®80 Solution) at Stage 17; C) T4 Embryo (Closed Control) at Stage 17; D) T5 Embryo (Injected With 0.01% Tween®80 Solution) at Stage 18; E) T2 Embryo (Sprayed With 1.5% HFFSRc) at Stage 19; F) T1 Embryo (Sprayed With 1% HFFSRc) at Stage 17. Final Magnification of 10x.

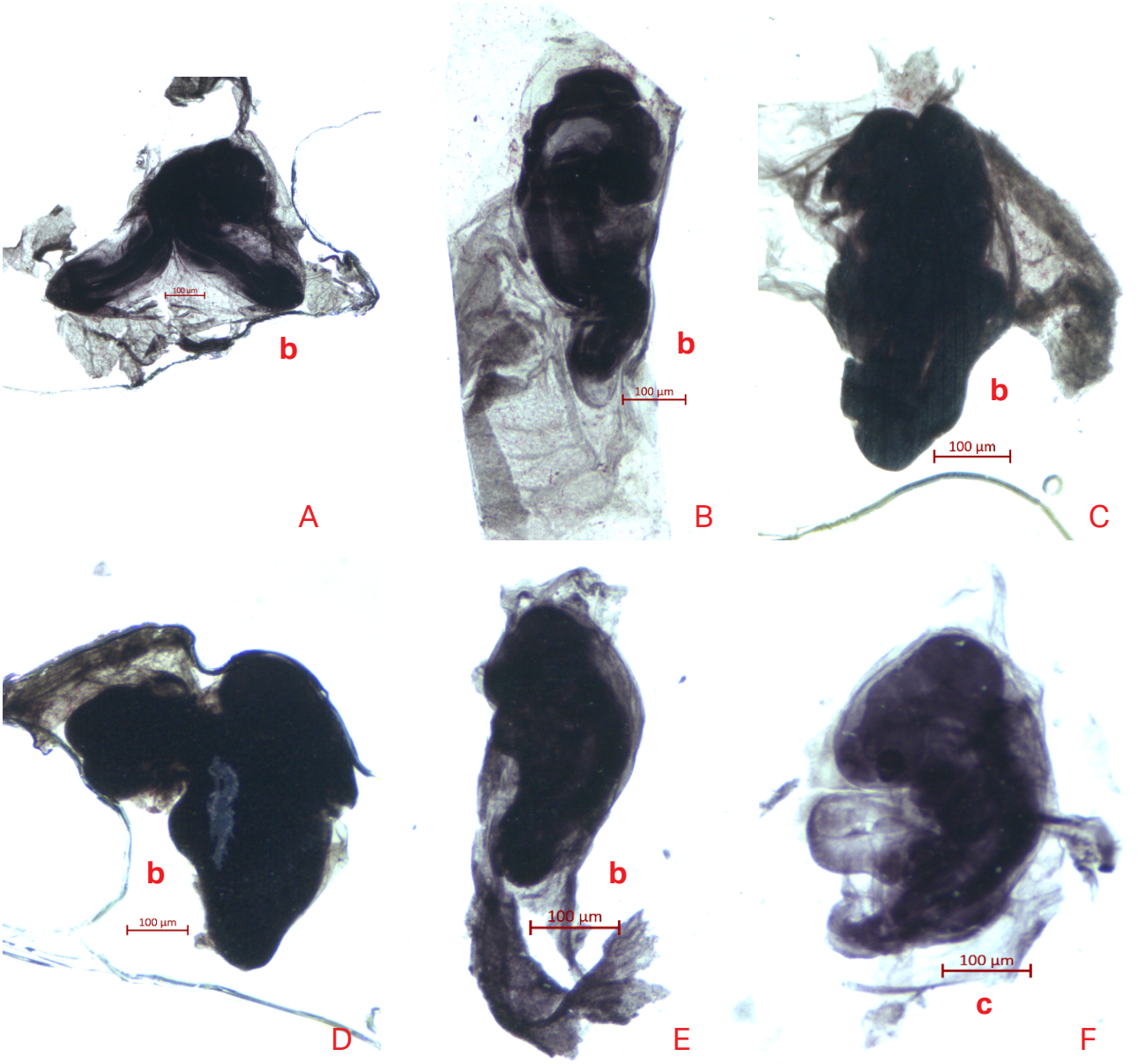

Figure 6. Stereophotography of Gallus gallus domesticus Embryos With Extensive Malformations. A) T4 Embryo (Closed Control) at Stage 17; B) T1 Embryo (Injected With 1% HFFSRc); C) T3 Embryo (Injected With 2% HFFSRc); D) T2 Embryo (Injected With 1.5% HFFSRc); E) T1 Embryo (Sprayed With 1% HFFSRc); F) T3 Embryo (Injected With 2% HFFSRc). Final Magnification of 10x.
Note. a = Gamma body (two bodies and a head), b = Totally malformed body, c = Body with caudal atrophy and failed division of the brain vesicles.

The majority of malformations found in all groups were those related to the body and caudal region of the embryos. Two main malformations were found: i) gastroschisis, a failure in the lateral and caudal closure of the body, and ii) caudal atrophy, characterized by

the shortening of the embryo's tail, this being the most common malformation. Flaws in the closure of the neural tract, called rachischisis, were also found (Figure 7).

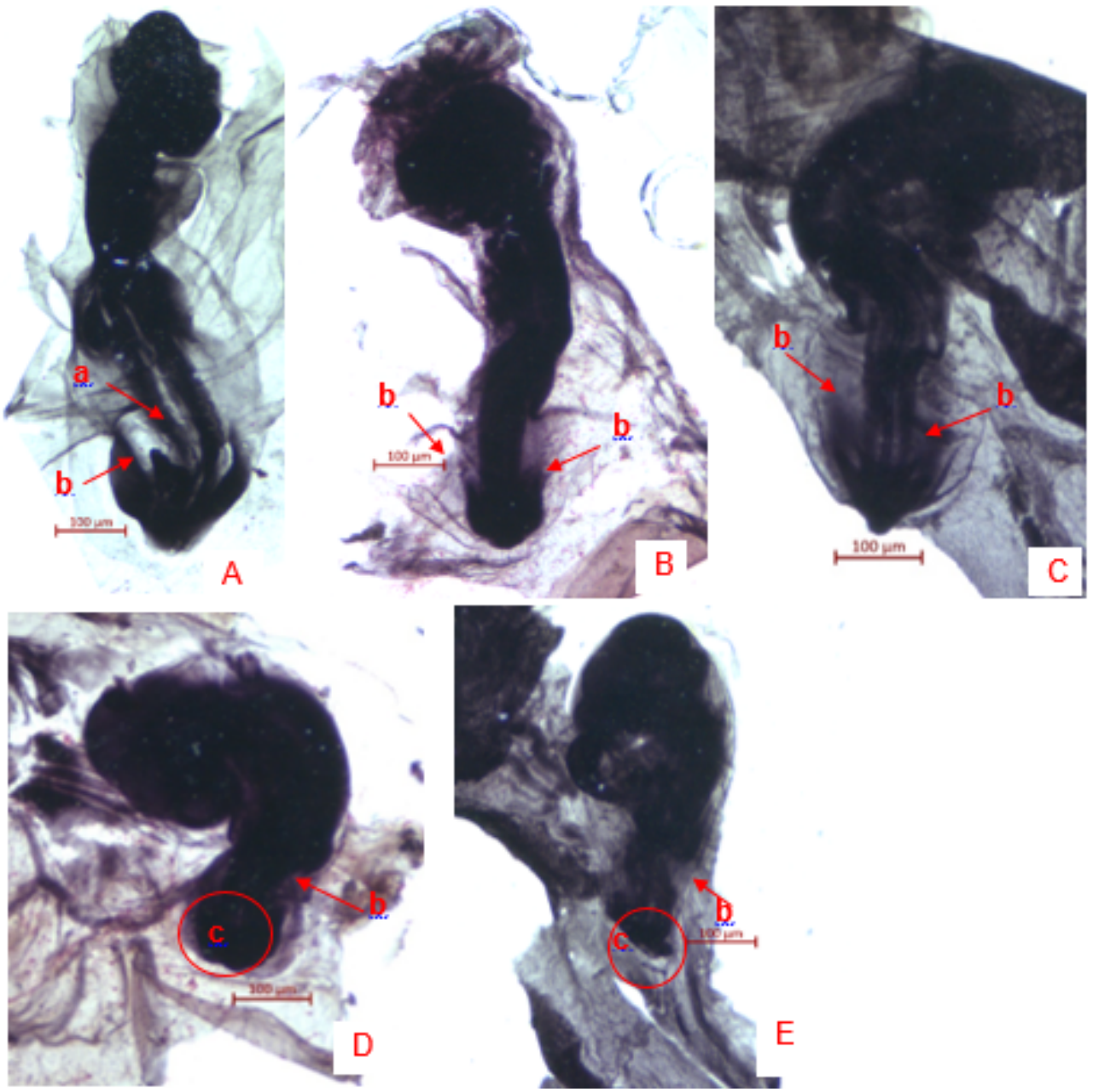

Figure 7. Stereophotography of Gallus gallus domesticus Embryos With Malformations in the Body and Tail Region.
A) T3 Embryo (Sprayed With 2% HFFSRc) at Stage 18; B) T2 Embryo (Injected With 1.5% HFFSRc) at Stage 17; C) T3 Embryo (Injected With 2% HFFSRc) at Stage 17; D) T1 Embryo (Sprayed With 1% HFFSRc); E) T2 Embryo (Injected With 1.5% HFFSRc). Final Magnification of 10x
Note. a = Body showing rachischisis, b = Body showing gastroschisis, c = body with caudal atrophy.

Discussion

Bird embryos are highly sensitive to the effects of potentially teratogenic external factors. Teratogenic factors are any substance, organism, physical agent, or deficiency present during embryonic or fetal development that causes changes in the offspring's structure or function (Dicke, 1989; Kmecick, 2017). Thus, the results obtained through the exposure of *G. gallus domesticus* embryos to possible teratogenic agents, such as HFFSRc, can highlight the effects of these agents on the embryonic development of vertebrates, which can be observed in embryotoxicology tests.

A critical factor for validating experiments with *G. gallus domesticus* embryos is their feasibility since, if there is a significant change in this, the experiment as a whole will be compromised. In this work, there were no significant changes in the feasibility of *G. gallus domesticus* embryos; the feasibility rate remained between 90 and 100%, values considered adequate for such experiments. Similarly, eggs of *G. gallus domesticus* exposed to mycelial growth filtrates of *Ganoderma lucidum* (Curtis) P. Karst. (Polyporales: Ganodermataceae) did not show significant differences in feasibility, with rates between 75% and 100% (Cruz et al., 2021).

On the other hand, HFFSRc reduced the survival rate of *G. gallus domesticus* embryos when injected into the egg chamber, a method commonly used in research with this biological model. A reduction in the probability of survival of *G. gallus domesticus* embryos was also observed in treatments involving the spraying of HFFSRc on eggshells, an innovative methodology used to simulate possible contamination of bird eggs and other vertebrates present in agroecosystems. Even so, the probability of survival was greater when compared to HFFSRc injection (internal contamination). Tests with other bioinputs, such as the plant extract of citronella, *Cymbopogon winterianus* Jowitt ex Bor (Poales: Poaceae), at its highest concentration, caused 50% mortality in *G. gallus domesticus* (Galvão et al., 2019). These values are similar to those observed in the present study when HFFSRc was injected into the egg chamber. Beauveria bassiana (Bals.-Criv.) Vuill. (Hypocreales: Cordycipitaceae), also caused mortality in embryos of the same species, reaching 30% (Larentis et al., 2019). In contrast to these, *G. gallus domesticus* eggs exposed to *G. lucidum* extracts did not show significant differences in survival between the extract and control treatments (Cruz et al., 2021).

The toxicity of the chemical compounds present in the HFFSRc fraction should also be considered. In this regard, it is important to highlight the presence of three flavones (naringenin, "prunin 3"-p-coumarate, and 5,6,2'-trimethoxyflavone), one fatty acid (ricinoleic acid), and one cinnamic acid (melilotoside). Naringenin can be considered toxic according to in silico studies using the ADMETlab 2.0 tool, which showed that it causes DNA fragmentation and may potentially cross the Caco-2 cell barrier (Macedo et al., 2024). Ricinoleic acid has shown toxicity in a study of the cytotoxic and genotoxic effects of its derivatives, where induction of apoptosis and necrotic cell death in animal tissues was observed (Blaszczyk et al., 2020).

Some botanical insecticides are considered teratogenic, and others are considered toxic to embryos. The extract of *Plectranthus barbatus* Andrews (Lamiales: Lamiaceae), applied in its hydroalcoholic format at the highest daily dose to pregnant female Wistar rats, caused delayed fetal development and an anti-implantation effect, which resulted in the death of the embryo. In addition, it caused delayed development in fetuses in the more advanced stages of pregnancy (Almeida & Lemonica, 2000). In another study, *Hyptis martiusii* Benth (Cidreira-Brava; Lamiaceae), when administered orally to pregnant rats, caused toxic effects in the pre-implantation and organogenesis phases (Caldas et al., 2020).

The assessment of heartbeats in vertebrates undergoing treatment is an indicator of the regulation of the autonomic nervous system, which has been associated with various somatic and mental disorders when unbalanced for long periods (Léonard et al., 2019). Therefore, factors that cause changes in heartbeat can cause numerous problems for the embryo. In the present work, 2% HFFSRc reduced the heartbeat of *G. gallus domesticus* embryos. Cruz et al. (2021) observed a similar reduction in heart rate when *G. gallus domesticus* embryos were exposed to *G. lucidum.*

The analysis of embryonic development through various stages allows us to determine whether exposure to different substances affects the normal growth pattern of embryonic structures (Kmecick, 2017). Embryos exposed to HFFSRc did not show changes in developmental stages when compared to those exposed to control treatments, suggesting that HFFSRc does not alter normal body organization. This is consistent with observations from studies where *G. gallus domesticus* embryos were exposed to the plant extract of *C. winterianus* (Galvão et al., 2019). Conversely, other agents, such as *G. lucidum,* have been shown to be toxic to *G. gallus domesticus* embryos, with exposure affecting the embryonic structure (Cruz et al., 2021).

Teratogenic agents can cause fetal malformations (Dicke, 1989). In *G. gallus domesticus,* a clear example of such agents is *B. bassiana,* which induced malformations in 66.7% of living embryos. These malformations included body and tail shortening and incomplete neural tube closure in the brain region. Additionally, patchouli essential oil from *Pogostemon cablin* Benth (Lamiales: Lamiaceae) caused malformations in 25% of living embryos, including caudal atrophy, tear duct dilation, rachischisis, and gastroschisis (Larentis et al., 2019). The plant extract of *C. winterianus* (Galvão et al., 2019) and the extract of *G. lucidum* (Cruz et al., 2021) also caused malformations in *G. gallus domesticus* embryos.

HFFSRc (1%, 1.5%, and 2%) can also be considered a teratogenic agent, as it resulted in a malformation probability ranging from 25% to 50% when injected into an egg's air chamber. However, when sprayed externally, the incidence of malformation was between 12.5% and 25%. It is important to note that some number of malformations are inherent to the embryonic development of any species (Carlson, 2014).

When HFFSRc is sprayed onto an egg, the eggshell acts as a barrier, protecting the embryo. The eggshell is formed in the isthmus, the region where the egg's internal and external membranes are created. The

inner membrane develops first, followed by the outer membrane, which is three times thicker than the inner membrane. Once complete, the eggshell is composed almost entirely of calcium carbonate (CaCO3), with small amounts of sodium, potassium, and magnesium. The two membranes remain together until the egg is laid, at which point they separate to form the air cell, which is crucial for the embryo's respiration (Figueiredo et al., 2021).

The air cell's primary function is to protect the internal contents of the egg against mechanical injuries and invasion by teratogenic agents, explaining the lower incidence of malformations when HFFSRc was sprayed onto the egg rather than injected. Additionally, it regulates gas exchange and water evaporation through the shell's pores and provides calcium for embryonic development (Sesti, 2003; Mazzuco, 2008; Figueiredo et al., 2021). It can be inferred that HFFSRc may be less harmful to oviparous vertebrates in the field, as they have a natural protective barrier against contaminating agents.

The malformations of *G. gallus domesticus* in this study were related to the cephalic, trunk, and caudal regions; failed closure of the neural tube was also observed. The malformations in the cephalic region were failures in the subdivisions of the brain vesicles and the closure of the neural tube (rachischisis). In other regions, malformations included caudal atrophy and gastroschisis in the abdominal region. In addition, many embryos presented general malformations that were considered serious, as they could possibly lead to the embryo's death. In humans, the main cause of infant mortality is congenital malformations (Moore et al., 2020). Congenital malformations exhibited at or after birth mostly originate before the eighth week of gestation (Sadler, 2012; Hill, 2023). As bird embryos aged three to five days are morphologically similar to human embryos aged three to five weeks of development, as well as having similar structures, it is possible to associate the malformations found in this work with malformations developing between the third and fifth week of human embryonic development (Gonzales & Macari, 2003).

Studies regarding the toxicity of teratogenic agents to the embryonic development of vertebrates have been increasing in recent decades. This research focus is justified due to the use of pesticides in agroecosystems, which may be linked to embryonic malformations or even deaths in non-target organisms. Therefore, it is necessary to carry out investigations that clarify the risks that these agents can cause to non-target organisms.

It is valuable to evaluate the effects of HFFSRc on *G. gallus domesticus* as this model is suitable for assessing the damage caused by botanical insecticides to vertebrates. Future tests analyzing the possible physiological, biochemical, and molecular changes caused by HFFSRc are recommended. It is worth highlighting the importance of carrying out tests with HFFSRc in other vertebrates to compare with this work's results and those of future work using *G. gallus domesticus* embryos as a biological model. We recommend using synthetic chemical insecticides or commercial botanical insecticides as a positive control in future tests, comparing these insecticides' previously defined degree of toxicity to that of HFFSRc.

The new methodology proposed in this work introduces the use of two exposure methods (injection and spraying), which results in a comprehensive assessment of the toxicity and impact of the tested compounds. Injection into the egg's air cell is a well-established method used in numerous studies (Galvão et al., 2019; Larentis et al., 2019; Cruz et al., 2021). It allows for an analysis of internal toxicity, simulating direct contamination of the egg's contents. In contrast, spraying represents an indirect exposure through the eggshell, similar to what would occur in the field. Comparing the effects of both exposure methods helps to understand the extent and mechanisms of toxicity better, enabling a thorough assessment of the risks and necessary mitigation measures to protect eggs and embryos of vertebrates in agroecosystems. This approach considers two fundamental parameters: the direct action of the agent on the non-target organism and, on the other hand, the physical protective barrier present in these organisms.

It is important to note the challenges of conducting tests with vertebrates, which makes the *G. gallus domesticus* embryo one of the most suitable alternatives for this purpose, given its life cycle. Therefore, standardizing new methodologies for exposing these embryos to contaminants such as the spraying technique used in this study, which differs from the injection method commonly used in studies with this model (Galvão et al., 2019; Larentis et al., 2019; Cruz et al., 2021) is crucial. This work contributes to the development of new testing methodologies for botanical insecticides on *G. gallus domesticus,* providing a form of exposure that more closely mimics natural conditions.

Certain application strategies must be adopted to prevent vertebrate eggs, such as those of *G. gallus domesticus,* from being affected by HFFSRc in the field. It is essential to consider the behavior of non-target animals within agroecosystems to define these strategies more effectively. The product should be applied away from native forests, legal reserves, and other protected areas. Additionally, it is crucial to carefully evaluate the environments where these products will be used to protect non-target organisms within these agroecosystems. Furthermore, given the toxicity of HFFSRc to *G. gallus domesticus* embryos, it is recommended that appropriate personal protective equipment (PPE) be used during application to ensure the safety of field applicators.

Conclusions

The hexane fraction of fruits and seeds of *R. communis* is toxic to *G. gallus domesticus* embryos, reducing their survival both when injected into the air chamber and when sprayed on the eggshell. This botanical insecticide also causes body malformations in *G. gallus domesticus* embryos when injected into the egg's air chamber.

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