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Sexing domestic chicken before hatch: A new method for *in ovo* gender identification

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ABSTRACT

Male chicks are an unwanted by-product when producing laying hens. The common practice to kill them directly after they have hatched gives rise to ethical concerns worldwide. The aim of this study was to develop an endocrine method to determine the sex of domestic chicken before hatch. On Days 7 to 10 of incubation, the allantoic fluid from brown layers' eggs (n = 750) was analyzed via enzyme immunoassay for their content of estradiol, estrone sulfate, and testosterone in order to detect gender differences. We successfully established a reliable method for *in ovo* sex identification on Day 9 of incubation by estrone sulfate measurement in the allantoic fluid. Female embryos displayed significantly higher hormone levels in the allantoic fluid than males (female: median = 0.312 ng/mL; male: median = 0.110 ng/mL; $P \leq 0.001$). Our method allows the sexing of domestic chicken at a very early stage of embryonic development, even before the onset of pain perception. The possibility to eliminate eggs containing male embryos on Day 9 of incubation represents a vast improvement compared with culling day-old chicks.

1. Introduction

Today, meat and egg production are highly specialized sectors of poultry industry with only little profit margins. The daily gains and feed utilization of male layers are less efficient compared with broiler chicken. This is the reason for the annual culling of about 40 million day-old male chicks in Germany; across the EU, 300 million male chicks are killed each year [1]. Considering animal welfare acts of Germany and Austria that permit animal killing only for good purpose [2,3], this unacceptable situation is a multinational trigger for ethical debates.

One way of solving this predicament is *in ovo* gender identification of domestic chicken and the accompanying possibility to eliminate the male embryos before they hatch. So far, two different approaches are being adhered to: gender identification of chicken embryos in the not incubated egg and gender identification in the incubated egg.

Recent studies report the sexing of fertilized chicken eggs before incubation by two different techniques. One detects gender differences in the DNA content via infrared spectroscopic imaging of blastoderm cells [4]. The other technique uses molecular biology methods to sex not incubated chicken embryos. Embryonic cell material is analyzed via polymerase chain reaction for the female-specific *Xho1* repetitive element [5] or the CHD-1 genes [6]. Nevertheless, all methods are not yet tested under *in ovo* conditions and, therefore, not ready for large-scale usage in hatcheries.

The methods described for gender determination in the incubated egg are on the basis of endocrine variances between males and females. In chicken embryos, first measurable gender differences manifest in their plasma levels of sex steroids on Day 7.5 of incubation [7,8]. Because of the individual localization of extra-embryonic vessels, the sampling of peripheral blood as described in previous publications [9,10] is not suitable as a standardized minimally invasive technique. Thus, the analysis of plasma is an inapt approach for identifying embryonic hormone differences in large-scale industrial usage.

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This study explores the allantoic fluid as a medium for endocrine gender determination. Sex steroids, which are secreted into the blood, get metabolized in the liver. After excretion via the kidneys, they accumulate in the allantoic fluid, the embryonic urine [11]. By analysis for gender-related hormones, one can therefore indirectly distinguish the embryonic sex. Advantageously, the allantois is easier to localize than extra-embryonic vessels. It starts to form on Day 3 of embryonic development and continues to enlarge directly under the eggshell until it encloses the complete egg's content [12].

In this study, we report a new method for *in ovo* sex identification of incubated domestic chicken eggs. To improve the animal welfare situation, we aimed to develop an endocrine method that allows gender determination before the onset of embryonic pain perception on Day 10.5 of incubation. The allantoic fluid of incubated brown layers' eggs was analyzed for sex steroids (estradiol, testosterone), as well as their metabolites (estrone sulfate) on Days 7 to 10 of incubation. The different endocrine markers were evaluated with regard to their suitability for gender identification according to their significance, practicability, and reliability.

2. Materials and methods

2.1. Incubation regime

For this study, fertilized eggs from Lohmann brown layers (Lohmann Tierzucht GmbH, Cuxhaven, Germany) were used (n = 975). Incubation took place in HEKA-Turbo 168 incubation and hatching devices (HEKA Brutgeräte, Rietberg, Germany). The eggs were set in incubators at 37.8 °C and 52% to 56% humidity with full automatic turning. On Day 18 of incubation, they were transferred into the hatcher, placing each egg individually in acrylic glass grids. Humidity increased up to 70% to 80% during hatching, as suggested by the egg supplier [13]. Just like natural brooding, hatching took place on Day 21 ± 1.

2.2. Sample collection

The allantoic fluid of chicken embryos was examined on distinct days of incubation. A preliminary study was conducted on Days 7, 8, 9, and 10 of incubation, and the allantoic fluid of 20 eggs was sampled and assayed for estradiol (E_2), estrone sulfate (E_1S), and testosterone (T). For the main study, which concentrated on E_2 and E_1S, a larger number of eggs were evaluated to substantiate the results observed in the preliminary study (Day 7: n = 150; Day 8: n = 150; Day 9: n = 300; Day 10: n = 150).

Before specimen collection, fertilization of the eggs was ascertained by candling. For sampling, two different egg positions were evaluated: position A (Fig. 1) and position B (Fig. 2). The allantoic fluid, being of a lower density than the embryo and the yolk sac, always accumulates at the highest possible point directly under the eggshell. At this localization, it was accessible for withdrawal. Incubating the eggs for 15 minutes at 37.8 °C in the appropriate position allowed the allantois to pool under the eggshell. The intended sampling localization (position A: highest point of the eggshell; position B: 0.5 cm next to the air chamber) was marked on the shell and disinfected with ethanol [14]. A 0.5-mm hole was drilled into this area using a drill fixed in a drill stand (Micromot 50/E, Micromot MB 140/S; Proxxon, Föhren, Germany). Allantoic fluid (20–50 μL) was sampled using an insulin syringe (Omnican 40; B. Braun, Melsungen, Germany) and immediately transferred into 1.5 mL polystyrene tubes (Eppendorf, Hamburg, Germany). Because sealing the hole does not improve the hatching rates [14], we refrained from this procedure. Seven days after each time point of sampling, the eggs were examined for a living embryo by candling.

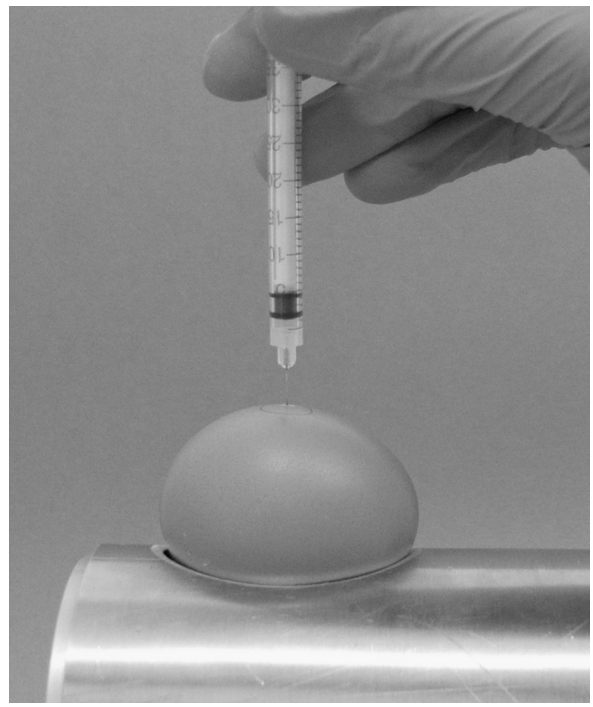

Fig. 1. Sampling of allantoic fluid in position A. The egg is placed horizontally, which causes the allantoic fluid to pool at the eggs highest point.

To evaluate the influence of sampling on hatching rate and embryo development, untreated control groups (n = 225) were hatched. The control eggs were set simultaneously in the same incubators as the eggs used for sampling the allantoic fluid.

2.3. Hormone analysis

The collected allantoic fluid was deep-frozen at −20 °C until further use. The samples were analyzed for E_2, E_1S, and T via competitive enzyme immunoassay (ELISA).

For the E_2 and T analyses, the allantoic fluid (15 μL) was diluted in ethanol (150 μL) and centrifuged. The supernatant was vaporized at 40 °C for 25 minutes and resuspended with buffer (sodium hydrogen phosphate, sodium chloride (both Merck, Darmstadt, Germany), bovine serum albumin (SERVA Electrophoresis, Heidelberg, Germany) dissolved in ddH_2O, pH 7.2). No preconditioning was conducted for E_1S analysis. Before assaying, the allantoic fluid was diluted with buffer.