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Review

A Review of Key Techniques for in Ovo Sexing of Chicken Eggs

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Abstract: The identification of chicken sex before hatching is an important problem in large-scale breeding applications in the poultry industry. This paper systematically reviews the key techniques for in ovo sexing of chicken eggs before hatching and presents recent research on molecular-based, spectral-based, acoustic-based, morphology-based, and volatile organic compound (VOC)-based technologies. Molecular-based methods are standard techniques for accurate sexing but require perforations by skilled technicians in certified laboratories to extract egg contents. Spectral-based techniques show great potential as noninvasive methods but require complex data processing and modeling. Acoustic-based techniques are sensitive to environmental noise. Morphology-based studies on the outer shape of the eggshell and distribution of blood vessels provide novel methods for in ovo sexing of chicken eggs. However, they face challenges such as the color, thickness, and smoothness of the eggshell. VOC profiling of chicken eggs allows sexing in the early stages of incubation; however, the VOC composition may be influenced by species or feed, and more research is required to explore potential applications. In addition, recent breakthroughs on in ovo chicken egg sexing are discussed. Physiological changes in chicken eggs during the whole incubation period have been well studied using metabolism and phenotype tools to enhance mechanism recognition. Furthermore, various sensing techniques, from the X-ray to terahertz range, and deep learning algorithms have been employed for data acquisition, processing, mining, and modeling to capture and analyze key features. Finally, commercialization and practical applications are discussed. This study provides a reference for in ovo sexing of chicken eggs before hatching in the poultry industry.

Keywords: in ovo sexing; molecular-based technique; spectral-based technique; morphology-based technique; VOC-based technique

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1. Introduction

In global poultry farming, the sex of chickens has a considerable impact on production performance and economic benefits. In terms of egg production, male birds cannot lay eggs and usually have a lower ratio of meat to feed than broilers. Male chicks are usually killed immediately after hatching since they are redundant in the industry and because male chicks will neither be suitable for egg production nor meat production. Ethical and animal welfare concerns about the culling of male chicks (approximately 7 billion each year [1]) have become an increasing issue in the egg industry; however, to date, no economically feasible in ovo sexing approaches for large-scale applications have been developed.

In large commercial hatcheries, sexing of newly born chicks is generally accomplished by three different methods according to the vent, color, or feathers of new hatching lines. Vent-based methods involve manual examinations of the reproductive organs and are not limited to specific breeds or crosses; however, these approaches require professional training to prevent disembowelment of the chick. In some breeds or strains, male and female chicks can be sexed according to their coat color or wing feather growth, which are determined by sex-linked genetic traits. These approaches are simpler and easier to explain to workers. However, these methods are still time- and labor-consuming. If sex can be identified at an early embryonic stage or even before incubation, male eggs could be used

as feed components, which is a safe and good circular approach to nutrient use. Moreover, fewer eggs would need to be incubated, which would reduce feed space requirements, CO_2 emissions, and energy consumption, which are all economically beneficial to farmers and the environment.

In recent decades, scientists and researchers have used various in ovo sexing strategies in chicken eggs before hatching or incubation. Figure 1 summarizes some invasive and noninvasive studies that have been researched for in ovo sexing of chicken eggs. These approaches can be divided into five major categories: (i) molecular-based techniques, (ii) spectral-based techniques, (iii) acoustic-based techniques, (iv) morphology-based techniques, and (v) volatile organic compound (VOC)-based techniques. Commercially applicable methods must be noninvasive, rapid enough for real-time applications, economically feasible, and ethically acceptable [2].

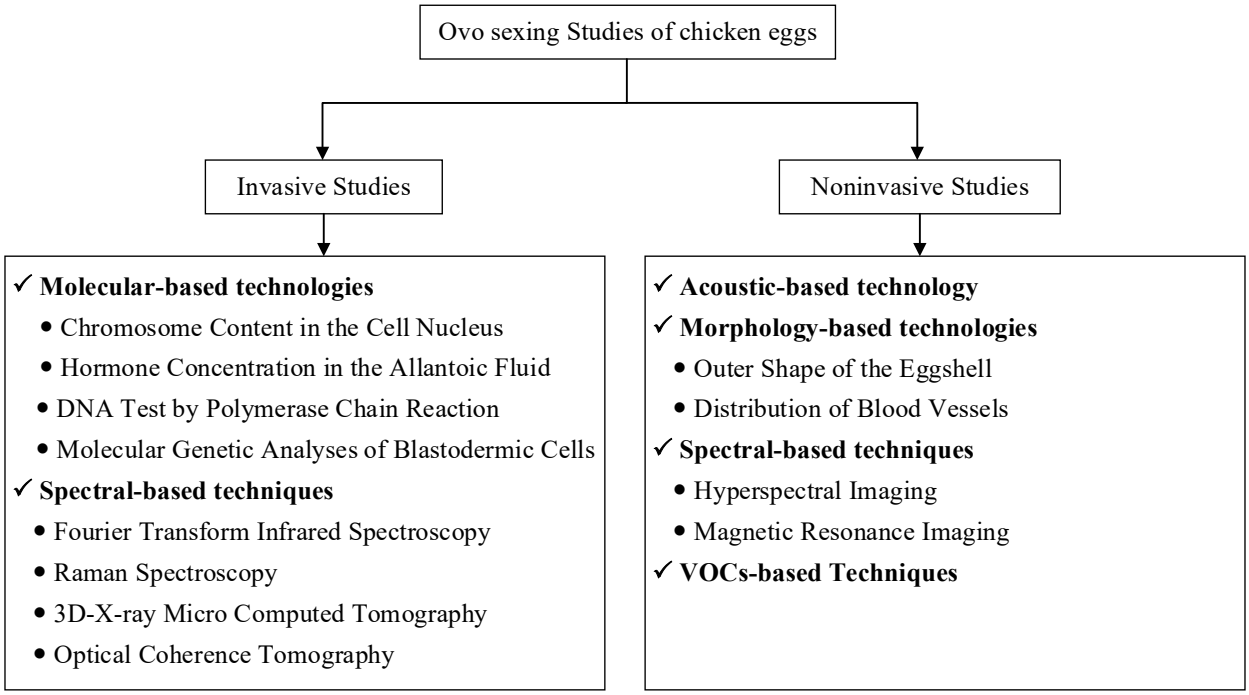

Figure 1. Studies on in ovo sexing of chicken eggs [3–19].

In this paper, five approaches for in ovo sexing of chicken eggs are described and analyzed. Their findings are a valuable source of information that can inform both conceptual and procedural knowledge for developing effective in ovo sexing strategies to prevent the killing of day-old male chicks. Next, breakthroughs in in ovo sexing of chicken eggs are discussed. Physiological changes in chicken eggs during the whole incubation period have been studied using metabolism and phenotype tools to enhance mechanism recognition. Moreover, various sensors, from the X-ray to terahertz range, and deep learning algorithms are suggested for data acquisition, processing, mining, and modeling to capture and analyze key features. Finally, commercialization and practical applications are discussed.

2. Research Progress of Chicken in Ovo Sexing Methods

In ovo sexing methods of chicken eggs are based on the initial assumption that male and female embryos exhibit specific characteristics (anatomical, physiological, molecular, and genetic) that should allow the differentiation of sexes during the incubation of fertilized eggs. In this section, five approaches for in ovo sexing of chicken eggs are described. The advantages and limitations of each method are also analyzed.

2.1. Molecular-Based Techniques

2.1.1. Chromosome Content in the Cell Nucleus

In birds, males have two identical sex chromosomes (ZZ), whereas females have heterogametic chromosomes (ZW). The difference in DNA content between male and female chicks is approximately 2% [20,21]. Flow cytometry is an essential tool for monitoring DNA content because the fluorescence emitted by each nucleus is directly proportional to the amount of DNA present. It can identify clear differences in DNA content between male and female cells caused by sex chromosomes. After the cells are broken, specific fluorescein is added and combines with nucleic acid. Then, laser irradiation is used to determine the DNA content in the nucleus according to different fluorescence intensities to determine in ovo sex in chicken eggs [3]. Flow cytometry is highly accurate; however, its low efficiency is more suitable for laboratory research than large-scale breeding applications.

2.1.2. Hormone Concentration in the Allantoic Fluid

Another molecular-based sexing method is the sampling of allantoic fluid during incubation, which begins to form at approximately the 5th day of incubation and reaches its maximum volume on the 12th to 13th day. Allantoic fluid is an excretory medium for the nitrogenous metabolites of avian embryos that contains embryonic cells, which allow embryos to be sorted on the basis of the presence of estrogenic compounds [22].

Estrone sulfate in the allantoic fluid can be measured to determine the in ovo sex of chicken eggs on the ninth day of incubation. Previous works have shown that the hormone level in male eggs is significantly lower than that in female eggs (Figure 2). This method can detect sex by the ninth day of incubation with an accuracy greater than 98% [4]. The fluid analysis requires 4 h and reduces the hatching rate by 3%. In a subsequent study, the method was tested on the layer strains Lohmann Brown (LB) and Lohmann Selected Leghorn (LSL) [23]. On day 9 + 4 h, the accuracy on the LB and LSL strains reached 98.7% and 100%, respectively; however, the hatching rate was reduced by 1.4% to 3.5% (LB) and 12.7% (LSL).

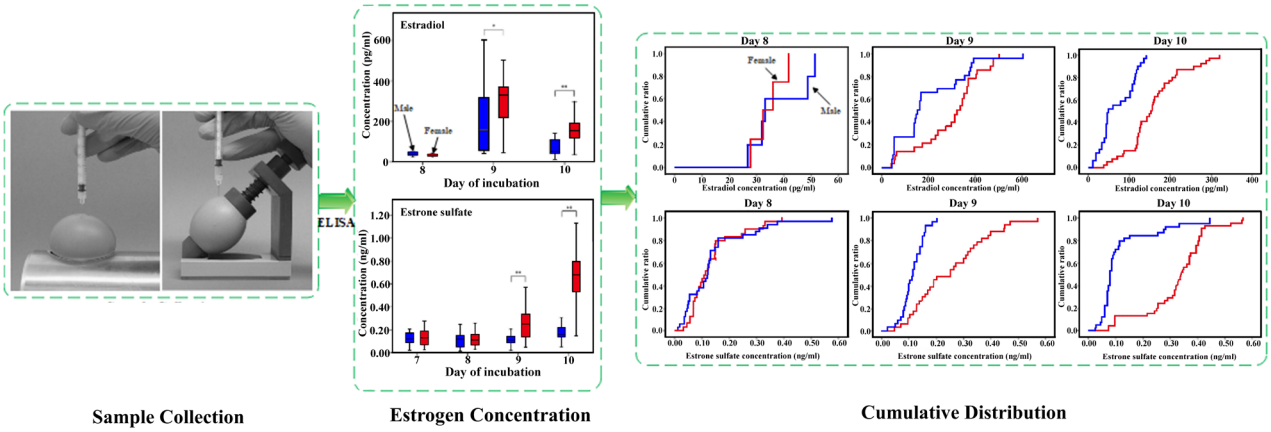

Figure 2. Flowchart of the evaluation of hormone concentration in the allantoic fluid [4]. (*: $p < 0.01$; **: $p < 0.001$).

2.1.3. DNA Test by Polymerase Chain Reaction

Polymerase chain reaction (PCR) is a common sexing technique used in avian field studies to amplify sex-specific genes (e.g., chromo-helicase-DNA-binding or CHD genes) located on the Z and W sex chromosomes [24]. The PCR-based method for in ovo sexing of chicken eggs was developed by Griffiths (1991) and improved by Clinton et al. (2001) [25,26]. Table 1 shows several examples of in ovo sexing studies based on PCR.

Agarose gel electrophoresis was used to screen PCR products and found that males had a single band (256 base pairs), while females had an additional second band (415 base pairs) [27]. Traditional PCR-based techniques are expensive and time-consuming because

they involve at least three steps: DNA separation, PCR, and gel electrophoresis. In recent years, an increasing number of studies have simplified PCR procedures, such as the development of real-time quantitative PCR (qRT PCR) and high-resolution melting (HRM). Furthermore, a "PCR-free" approach was applied to cleave nucleic acid molecules at specific sites based on structure rather than sequence [5].

Table 1. Examples of in ovo sexing studies based on PCR.

Categories	Samples	Methods	Results	Reference
Nuclear DNA content in the cell	Blood samples from birds	The flow cytometric technique was used to estimate the nuclear DNA content of erythrocytes in blood samples.	The samples were stored at 4–20 °C and successfully analyzed after at least five days. The flow cytometric technique can be completed in a few minutes.	[3]
Hormone concentration in the allantoic fluid	Allantoic fluid from eggs	Allantoic fluid was collected and estrone sulfate concentration was measured on day 9 of incubation, showing that male embryos displayed significantly lower hormone levels than female embryos.	This method can detect sex differences by day 9 with an accuracy greater than 98%. The fluid analysis required 4 h and reduced the hatching rate by 3%.	[4]
DNA test by PCR	Blood from ISA brown chicken eggs	The Hologic Invader® sexing assay, which includes a W-rpt/CR2 probe mixture and core reagent kit, was developed to monitor fluorescence.	This assay can distinguish the sex of individual animals with as little as 1 ng of DNA or 125 nl of whole blood or as few as 250 cells.	[5]
	ISA brown, Dekalb white, Bovan brown, and Shaver black eggs	The Q-PCR technique was used to incubate beads chelated with potential PCR inhibitors followed by centrifugation to identify genes on chromosomes W (SWIM and Xho-I) and Z (DMRT).	The Q-PCR method exhibits 100% concordance and specificity for the in ovo sexing of 176 embryos.	[28]
	Fertilized white Leghorn chicken eggs	The qRT–PCR technique was performed on mRNA from chick gonads and other tissues.	The qRT–PCR results of each sample agreed with those obtained by morphological examinations and PCR analyses.	[29]

2.1.4. Molecular Genetic Analyses of Blastodermic Cells

After an egg is laid, a small germinal disc of cells (4–5 mm) called the blastoderm can be found on the surface of the yolk [30], which contains information on whether the chick is male or female [31]. Various imaging methods, such as 3D X-ray microcomputed tomography (Micro-CT) and magnetic resonance imaging (MRI), have been used to localize this region [6].

Molecular-based methods achieve the best accuracy and sensitivity among the various chicken egg in ovo sexing methods. With in-depth research on sex-linked genes, PCR has been widely used in various fields and is not affected by the chicken breed. However, the extraction of samples from other organ tissues may be destructive and can severely impact embryo development. To obtain reliable and accurate results, PCR requires consumable reagents (e.g., sequence-specific primers) that must be tailored to detect specific pathogens. Due to their elaborate procedures, especially during sample preparation (collection and extraction), PCR techniques are more suitable for laboratory studies than farm applications. Comparatively, accurate sampling of allantoic fluid for analysis reduces the risk of embryo harm. In ovo sexing of chicken eggs via analysis of estradiol, estrone sulfate, or other biomarkers in the allantoic fluid on day 9 of incubation is another alternative approach.

2.2. Spectral-Based Techniques

2.2.1. Fourier Transform Infrared Spectroscopy

As another in ovo sexing method, spectroscopy-based techniques can measure differences in DNA [32,33]. Fourier transform infrared (FT-IR) spectroscopy uses a mathematical process (Fourier transform) to translate raw data (interferogram) into actual spectra. The infrared transmission and absorption spectra can provide molecular information about avian sex based on pulp cells extracted from the feather shaft [33].

The in ovo sexing of chicken eggs using FT-IR spectroscopy is already feasible in newly laid chicken eggs due to the germinal disc, which contains 40,000 to 60,000 blastoderm cells [7]. A small number of cells can be extracted from the eggs to assess the difference in DNA content between male and female embryos. Spectroscopy-based techniques rely on locating the blastoderm and inspecting the chemical makeup of its cells. The hatching rates of nonincubated and incubated eggs differ considerably after FT-IR spectroscopy [8]. Eggs that were incubated for up to 72 h in this study hatched at higher rates. Moreover, compared with the shortest egg incubation time (24 h), the hatching rates of nonincubated and freshly laid eggs were reduced by 6.6%. In contrast, the same manipulation had less effect on embryo development after 72 h of incubation, with a hatching rate of 80.9%. Therefore, the FT-IR process must be carried out carefully, as blastoderms are delicate and sensitive to the environment.

2.2.2. Raman Spectroscopy

Raman spectroscopy is unique for each molecule and is often referred to as the "molecular fingerprint" in rapid elemental and molecular analyses in a wide variety of applications. However, Raman spectroscopy differs from FT-IR in terms of the method for identifying cell composition, which prevents damaging cells on contact. Damage to living cells can be prevented by choosing near-infrared (NIR) excitation wavelengths (e.g., 785 nm), as NIR photons do not have sufficient energy to induce molecular changes.

NIR Raman spectroscopy was used to identify differences in nucleated blood cells [9]. Moreover, spectroscopic analyses of NIR fluorescence signals of blood flowing in extraembryonic vessels can discriminate male and female chicken eggs [10]. Male embryos have a stronger median fluorescence signal than female embryos, which may be due to the higher density of blood cells observed at days 13, 15, and 18 of incubation [34]. The influence of the supravascular membrane on the blood fluorescence signal intensity and Raman spectrum was also analyzed, and a shell-scattering correction method was proposed to compensate for the variable signal loss caused by Rayleigh scattering due to different inner membrane thicknesses. The hatching rates of eggs with 10 mm and 12 mm diameter shell windows at the pointed end, which were created using CO2 lasers, were determined to be 95% and 91% compared with those of eggs without perforations [11]. Figure 3 shows a flowchart of in ovo sexing of chicken eggs using Raman spectroscopy.

2.2.3. Hyperspectral Imaging

Hyperspectral imaging methods use sensing systems capable of plotting many closely spaced wavelengths. Hyperspectral-based methods for in ovo sexing of chicken eggs cover the near-ultraviolet region (300–380 nm), visible region (380–780 nm) and near-infrared region (780–1500 nm).

To determine in ovo sex in breeder eggs, VIS/NIR spectra of layer lines with gender-specific down feather colors was used, achieving an overall accuracy of 97% for 14-day-old embryos [17]. A VIS/NIR transmission spectral acquisition system was constructed and data in the wavelength range of 500–900 nm were used for correlation analyses [35].The best recognition effect was found at day 7 of incubation. An 870 nm NIR sensor was also applied to calculate the opacity of the ratio of the input LED current to the average output voltage [36]. The opacity was found to be relatively low until day 10 of incubation; the opacity then increased significantly on days 12 to 16 and was sufficient to distinguish sex. This approach obtained an accuracy of 84.0% on incubation days 16 to 18. Similarly, the ra-

tio of two wavelengths in the longitudinal visible light transmission spectrum (T575/T598) was used to monitor sex-specific embryonic development rates based on the production of hemoglobin pigment in red blood cells [37]. Visible-near-infrared (visNIR) point spectroscopy range from 300 to 1145 nm was used to individually illuminate Isa Brown eggs on the 8th to 14th day of incubation, resulting in a prediction accuracy of 99.52% on day 14 [38]. A dual in ovo fluorescence excitation at 532 nm and 785 nm excitation was used to identify the sex of the embryo after a few days of incubation [39]. It is shown that the observed sex-related differences in the fluorescence intensities were based on the embryonic hemoglobin synthesis. The accuracy of sex determination was 96% for both sexes.

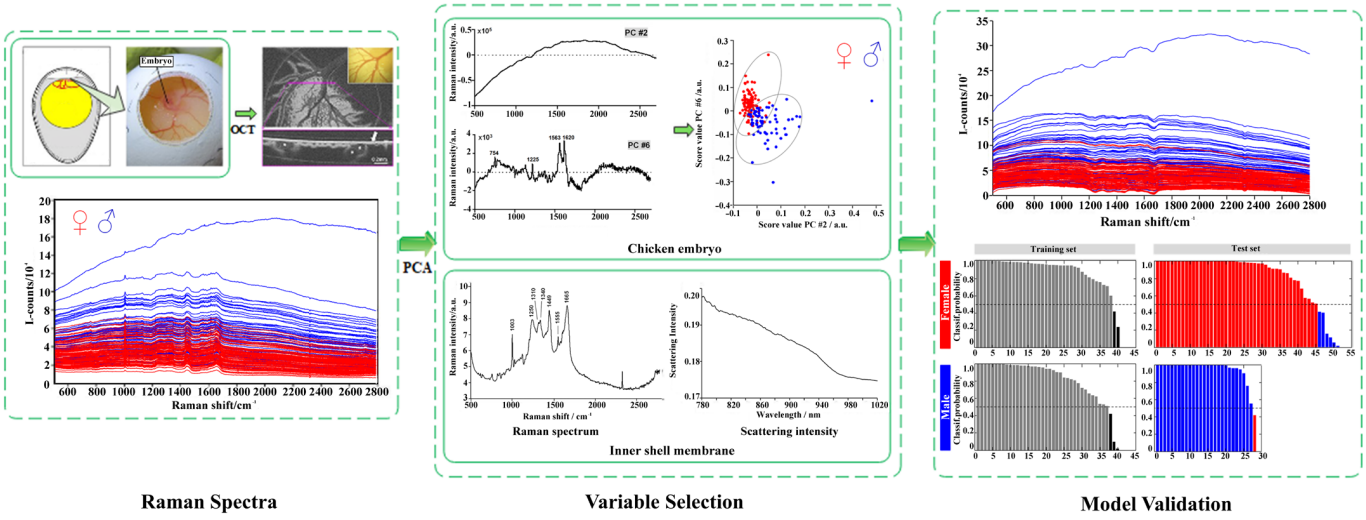

Figure 3. Flowchart of Raman spectroscopy of chicken egg in ovo sexing [11].

2.2.4. 3D X-Ray Microcomputed Tomography

Three-dimensional X-ray microcomputed tomography (Micro-CT) is a 3D imaging technology that uses X-rays to visualize the interior of an object slice by slice. Molecular genetic analysis of dermal blastocysts can be used for in ovo sexing of unincubated eggs. However, knowledge of the precise location of the germinal disc is critical for carefully guided cell biopsies. In principle, microcomputed tomography is a suitable method for determining the position of the germinal disc in unincubated oocytes without damaging the eggshell. No negative effects on embryonic development or the hatching rate of irradiated hatching eggs have been identified [12].

2.2.5. Optical Coherence Tomography

Optical coherence tomography (OCT) is an optical imaging technique that provides real-time 3D images of micron-scale tissue structures. In 2010, OCT was shown to be a suitable method for imaging and localizing the germinal disc in eggs [6]. Moreover, a spectral-domain OCT system was applied to acquire 3D OCT and microscopy camera images to locate perfused extraembryonic vessels while leaving the inner eggshell membrane intact [11].

2.2.6. Magnetic Resonance Imaging

A noninvasive method for in ovo sexing of domestic chicken eggs was developed based on magnetic resonance imaging (MRI) [18]. In addition to the embryo, MRI sequences, particularly T1-weighted images, can clearly distinguish the protein, yolk sac, allantoic sac, and amniotic cavity. Table 2 shows several examples of optical-based methods for in ovo sexing of chicken eggs.

Table 2. Examples of studies on sexing of eggs using spectroscopic and imaging techniques.

Types	Samples	Incubation Age	Band Range	Data Processing and Modeling	Accuracy (%)	Reference
FT-IR	Blastoderm cells	>3 d	1790 &1000 cm^{-1}	Removal of outliers and linear two-point baseline corrections	Not specified	[7]
Raman	White eggs	3.5 d	785 nm	PCA, genetic optimal region selection, and nonlinear discriminant analysis	91	[11]
Transmission	Brown eggs	14 d	600–950 nm	PCA and a linear discriminant analysis model	97.47	[17]
Hyperspectral	White eggs from brown chickens	10 d	600–900 nm	SVM, PLSDA, and ANN	SVM: 75 PLSDA: 75 ANN: 82.86	[40]
	Jing No. 1 chickens	7 d	360–780 nm	MSC, CARS, SPA, and GA-ELM	87.14	[41]
	Guo Shao No. 1 chickens	7 d	500–900 nm	SPA+CNN	93.83	[35]

Spectroscopy and imaging technologies are noncontact, nondestructive techniques that allow for invasive and noninvasive in ovo sexing of chicken eggs. Spectroscopy-based techniques show extraordinary potential for rapid and noninvasive in ovo sexing of chicken eggs. The presented optical method showed that in ovo sexing can be performed within 3–4 days, and the accuracy rate reached 93%, which greatly reduces incubation times and costs [10]. In some recent studies, the egg contents were extracted to determine the spectral reflectance or transmission characteristics, achieving an overall accuracy of 91% [11], while other studies analyzed the transmission spectral features of whole eggs. The former approach is more accurate and enables sexing of chicken eggs on the third day of incubation, while the latter technique can achieve in ovo sexing as early as the ninth day of incubation. The whole-egg-based spectral analysis approach is promising because the egg is not destroyed, which is more acceptable to most farms and simplifies the system development and integration. With the advantages of high precision and short analysis times, the optical-based approach utilizes early embryonic stages for analyses. However, this method has not yet shown clear patterns due to the lack of mechanistic studies.

2.3. Acoustic-Based Techniques

The heart rate of the egg is a kind of embryonic heartbeat signal that begins on days 2 to 3 of incubation. The heartbeat frequency of normally hatched eggs is not fixed after the ninth day of incubation and ranges from 1 to 4 Hz [42]. Heart rate data from 8 to 12 o'clock on days 15–19 of incubation were measured using a lie detector recording system [13]. The results showed that the average heart rate of female embryos was 2–4 times/min higher than that of male eggs after 17 days of incubation. Due to the high stiffness of the eggshell, the heartbeat signal passing through the eggshell is generally very weak. However, optical detection methods involving strong light transmission can be applied to measure the heartbeat signals of egg embryos. All these methods are sensitive to environmental noise.

The feasibility of in ovo sexing based on sound characteristics in poultry farms has not yet been explored. Methods for preprocessing environmental noise while collecting the heartbeat signals of poultry eggs have not been studied. Thus, acoustic-based in ovo sexing of chicken eggs is still far from a practical application.

2.4. Morphology-Based Techniques

2.4.1. Outer Shape of the Eggshell

One chicken egg in ovo sexing method utilizes the shape characteristics of eggshells. This rapid and noninvasive method can be applied before egg embryos are incubated. Table 3 shows four studies on the relationships between outer eggshell shapes and sex.

Table 3. Morphological egg sexing techniques.

Categories	Samples	Methods	Results	Reference
Outer shape of the eggshell	White eggs	The egg width (W), length (L), weight, and volume were measured to determine the sex of the fertilized white eggs before incubation. The egg volume V and shape index SI were estimated according to these measurements as follows: $SI = (W/L) \times 100$ $V = (/6)L \times W^2$	A multiple logistic regression model was built to determine the sex of the hatching chick. The female chick sex probability (IP1) was calculated as follows: IP1 = −0.39531+(0.01214 × SI) (R2 = 0.25). Eggs with greater SI values (p = 0.001) and lower volumes (p = 0.004) were more likely to produce female chicks.	[15]
	Duck eggs	The eccentricity (Ecc) of each sample was used to determine a specific threshold value to separate male and female eggs. $ecc = \frac{\sqrt{(L/2)^2-(W/2)^2}}{L/2}$	The results show that with an eccentricity threshold value of 0.6441, the duck egg sex prediction accuracy reached up to 86%.	[43]
	Japanese quail eggs	The W, L, SI, Ecc, area, geometric mean diameter (Gd), and sphericity were measured and calculated to establish an in ovo sexing model for the eggs. $Gd = \left(\mathrm{LW}^2\right)^{1/3}$ Sphericity $= (Gd/L) \times 100$	The Gaussian naïve Bayes model is the best classifier for data using two features (Ecc and SI), achieving an average accuracy of 82.88% (males: 85.14% and females: 80.16%).	[44]
	Turkey eggs	The combination of plumage color, physical external egg characteristics, the color of down feathers, and behavioral approaches to determine sex of turkey eggs.	The specificity values were found to be 49.12, 93.33, and 100%, while the sensitivity values were observed to be 74.64, 91.03, and 100%, which translated into accuracy of 63.10, 92.26, and 100% in black, black-roan, and bronze-roan poults, respectively.	[45]
Distribution of blood vessels	Jingfen No. 1 eggs	The 11-dimensional feature parameters of the image were extracted with difference box, gray level co-occurrence matrix, gray histogram, and geometric methods. A GA-BP-based neural network was established for in ovo sexing of chicken eggs on day 4 of incubation.	The classification accuracies of the training and prediction set were 99.73% and 82.80%, respectively.	[16]
	Jingfen No. 1 eggs	The 2916-dimensional fully informative image features were extracted through a gray horizontal co-occurrence matrix with 5-dimensional features and histogram of gradients (HOG) orientation. In addition, the 96-dimensional features were simplified using sampling and PCA dimensionality reduction-gray co-occurrence matrix methods. Support vector machine (SVM), backpropagation (BP) neural network, and deep belief network (DBN) models were constructed.	The DBN model had the highest accuracies of 76.67% (male) and 90% (female), with an average accuracy of 83.33%. The DBN model had the longest discriminant time of 7.8 s.	[46]

The maximum length and diameter of 1223 chicken eggs were used in a genetic comparison of female and male eggs [14]. The results showed that the outer shape differences could not be correlated with sex. In another similar study, the sex of fertilized white layer eggs was determined by morphological measurements [15]. The results showed that the egg shape index, egg length, egg width, and egg volume differed significantly between male and female eggs, while the egg weight and replicate number did not differ significantly depending on the sex of the hatching chick. The authors assumed that morphological measurements of prehatched eggs might be an indicator of the sex of hatching chicks. In addition, morphometric techniques have been applied to duck and Japanese quail eggs.

The eccentricity was adopted to analyze the roundness of duck eggs [43]. The eccentricity threshold was set as 0.6441, and eggs with an eccentricity less than the threshold were regarded as female, while eggs with an eccentricity equal to or larger than the threshold were regarded as male. The prediction accuracy of this method reached 86%. In another study, the morphological characteristics (eccentricity and shape index) of Japanese quail eggs were recognized as important factors by image processing technology and edge detection models, obtaining an average accuracy of 82.88% [44]. It can be seen that the correlation between eggshell morphology and sex may not be strong. The accuracy of an in ovo sexing model dependent on shape parameters is still too low to be accepted by poultry farms. Sex determination was performed using 15 methods, which included testing external egg metrics and eggshell color, poult morphological appraisal and phaneroptics, and behavioral traits for Andalusian turkey poults [45]. The results suggest that the method combination tested in this study could be considered a highly accurate, simple, and affordable alternative for sex determination in turkeys.

2.4.2. Distribution of Blood Vessels

Some studies have shown that RGB images acquired under LED light sources on the fourth day of incubation can be used to distinguish male and female eggs. To facilitate observation, a machine vision image acquisition system must be constructed to obtain RGB images of eggs under an LED light source. Moreover, several preprocessing algorithms need to be performed to extract the blood vessel characteristics.

To date, morphometric methods have used the distribution of blood vessels to recognize white male and female eggs on the 4th day of incubation. In the image acquisition system shown in Figure 4, RGB images of Jingfen No. 1 white eggs were collected on days 4 to 9 of incubation and analyzed [16]. Then, a gender identification model was established based on the genetic algorithm backpropagation (GA-BP) neural network. The 11-dimensional characteristic parameters used in the model include 10 blood vessel texture description parameters and 1 geometric parameter. The accuracy of this method reached 82.80% on the 4th day of incubation. In another study, a histogram of oriented gradients (HOG) and principal component analysis (PCA) were applied to extract egg features from RGB images and reduce the dimensionality [46]. A deep confidence network (DBN)-based model was constructed, obtaining a recognition rate of 83.33% and a discrimination time of 7.8 s. The blood vessel distribution method is more suitable for white eggs with good light transmission, and the best time to observe the bloodline is on the 3rd–5th day of incubation. In addition, different eggshell qualities (e.g., sandy shell, dark-spotted) may also affect the detection of blood vessel images.

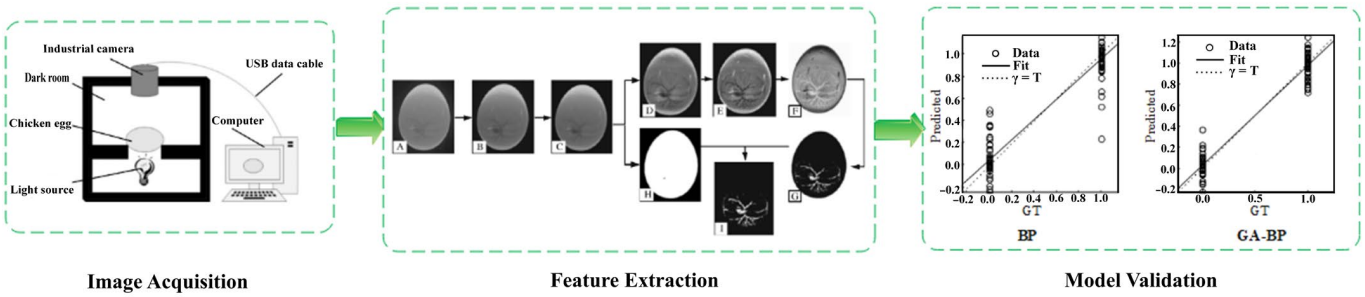

Figure 4. Flowchart of the distribution of blood vessels in in ovo sexing [16].

Morphology-based techniques based on the outer shape of the eggshell and distribution of blood vessels are rapid, low-cost, and noninvasive in ovo sexing methods. The blood vessel distribution can be used to identify the sex on day 4 of incubation, while the outer shape features can be used to determine sex before hatching, greatly reducing incubation costs. Morphology-based in ovo sexing methods that utilize regular cameras are more economical than those that use expensive chemical analysis instruments. However,

the accuracy of these methods is approximately 80–85%, which is less than the accuracy of molecular and spectral techniques. Nonetheless, the noise in the extracted texture features reduce the accuracy of the model. Furthermore, the model has not been applied to chicken eggs with brown shells or eggs with eggshell or calcium spots. Multiple sensors can be combined to address this problem. In future studies, more effective denoising methods should be adopted to eliminate the effect of noise on image information while retaining the image information to improve the discrimination accuracy and reduce the discrimination time.

2.5. Volatile-Organic-Compound-Based Techniques

In recent years, various studies have attempted to determine whether eggs are fertilized and to identify males and females by detecting changes in VOCs from chicken eggs. However, little work in the literature has focused on the odor attributes of chicken eggs, except in terms of freshness [47,48]. 2-Undecanone is a volatile ketone that differs between male and female chicken eggs and has previously been identified as a hormone-related component of avian odor [49]. VOCs from hatched Japanese quail eggs can indicate the gender and developmental status of the embryo [50]. Specifically, VOCs from eggs change during incubation, and sex can be predicted as early as day 1 of incubation.

Solid-phase microextraction (SPME) and gas chromatograph-mass spectrometry (GC-MS) combined with electronic nose (E-nose) have been widely investigated for their ability to detect VOCs from food matrices. A total of 111 different volatile compounds in egg yolks were identified by using headspace solid-phase microextraction (HS-SPME) and GC-MS [51]. VOCs released from chicken egg sin the first half of incubation were analyzed [52]. In the experiment, each egg was incubated individually at 37.7 °C by placing the egg in a closed glass jar for one hour, followed by 70 min of extraction using a solid-phase microextraction fiber. Then, the fiber was desorbed, followed by a 1 h GC-MS analysis. Extensive identification resulted in the appearance of 65 compounds. Similarly, VOCs emitted by unfertilized, infertile, and fertile eggs were collected by SPME-GC-MS combined with E-nose, and characterized the VOCs to explore potential biological information and fertilization-specific VOCs [19]. A total of 14 volatiles were identified in unhatched white Leghorn eggs, including nonanal, decanal, 6-methyl-5-hepten-2-one, and 6,10-dimethyl-5,9-undecadien-2-one. Furthermore, the potential of SPME/GC-MS as a nondestructive tool was investigated for characterizing odor differences between male and female chicken eggs on the ninth day of incubation [53]. A total of 18 VOCs were identified in hatched eggs. The results showed that nonanal, cedrene ($p < 0.05$), heptanal (area, $0.05 < p < 0.1$), carbon dioxide (area, $0.01 < p < 0.05$), 1-heptadecanamine, and undecanal (area, $0.05 < p < 0.1$) were significantly positively correlated with the sex of the eggs on days 1 and 5 of incubation, respectively. Moreover, 6,10-Dimethyl-5,9-undecadien-2-one (area, $0.05 < p < 0.1$), octanal (percentage, $0.01< p < 0.05$), and octanal (percentage, $0.05 < p < 0.1$) were significantly negatively correlated with the sex of the eggs between days 7 and 9 of incubation.

VOC profiling is a new and promising approach for incorporating potential biological information for in ovo sexing of chicken eggs [52]. Some studies have shown that the odor composition of eggs differs significantly [53]. Eggs should be maintained in individual septa jars during incubation to prevent contamination of VOC measurements. Measurements for individual eggs were performed using PTR-MS equipment according to raw VOC data and were processed using analysis software to calibrate and determine the volatile compounds present in the samples. Machine learning methods were then used to identify patterns between sexes. As incubation progresses, the VOCs produced as a direct result of embryonic metabolism result in divergent volatile compositions between male and female eggs. This noninvasive method of detecting the odor of hatching chicken embryos has crucial research value in the field of chicken egg in ovo sexing. Moreover, detecting the odors of hatching eggs is noninvasive, and collecting and characterizing the odor has considerably less impact on the egg embryos than spectral irradiation techniques. However, due to the large individual variability in the VOCs, which are affected by many factors, such as breed, diet, and the limited number of samples tested, the sensitivity of the technique is

not very high for some compounds. Additionally, most importantly, efficient acquisition of VOCs per egg requires hardware improvements and breakthroughs; further research is needed on how to develop intelligent equipment that integrates with actual hatchery facilities. To improve the accuracy of noninvasive odor identification for chicken eggs, more sensitive analytical methods, such as SIFT-MS, should be used in the future to extend the egg odor detection range, optimize the model parameters, and build deep neural models with higher prediction accuracy for sexing chicken eggs.

3. Breakthroughs and Perspectives

According to recent research, the sensory nervous system initially forms on the seventh day of incubation [1], which suggests that the in ovo sex must be determined earlier than that moment. Recent research progress on in ovo sexing of chicken eggs, including intelligent sensing methods, data preprocessing techniques, model construction approaches, and different application levels, is discussed and analyzed. The physiological changes in chicken eggs during the whole incubation period have been studied using metabolism and phenotype tools to enhance mechanism recognition. Moreover, various sensors, ranging from the X-ray to terahertz range, and deep learning algorithms, have been employed for data acquisition, processing, mining, and modeling to capture and analyze key features. Finally, commercialization and practical applications are discussed.

3.1. Mechanisms: Analysis of Physiological Changes in Chicken Eggs during Incubation

Sex differentiation is the process by which genetic sex is transformed into phenotypic sex through the actions of hormones and hormone receptor genetic signaling pathways under certain environmental effects. The phenotypic sex of eggs is determined by a combination of genetic and environmental factors in somatic cells [54]. Various sensing and classification methods could be used for the in ovo sexing of chicken eggs according to the distinct physiological characteristics of different incubation stages. Figure 5 shows several embryonic development stages in chicken eggs during incubation.

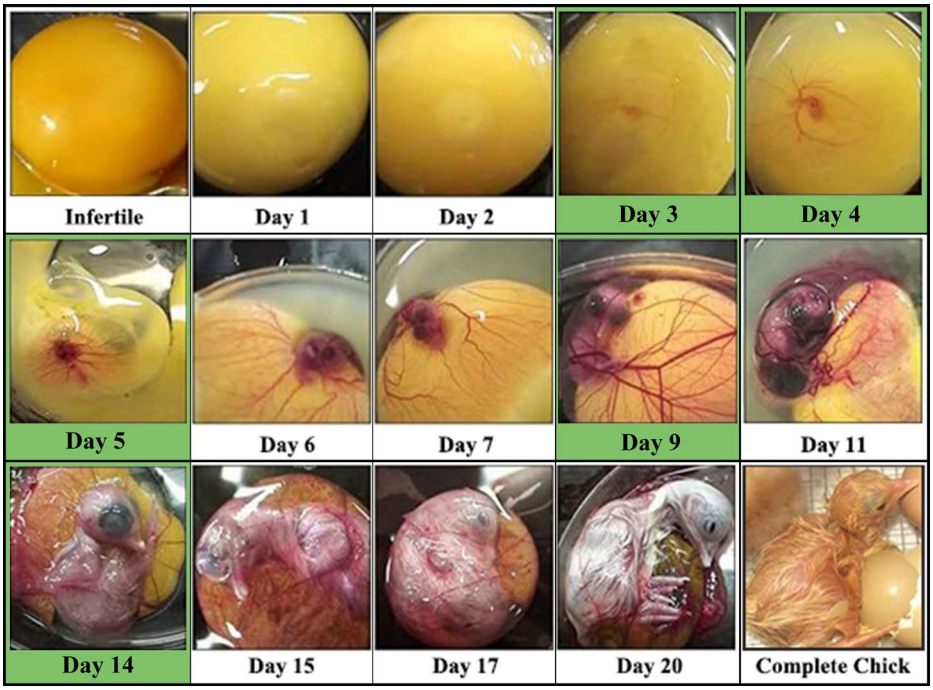

Figure 5. Chicken embryo development.

After incubation begins, a pointed thickened layer of cells can be observed at the tail or end of the embryo. This region, which is known as the germinal disc or blastoderm,

is the primitive streak and longitudinal axis of the embryo. The germinal disc consists of the germinal vesicle of the oocyte and the surrounding cytoplasm, and egg sex can be determined by spectroscopy and other methods in this region in the unincubated state [6,31]. On the second day of incubation, the blastoderm region grows, and blood islands begin to link, forming the vascular system, while the heart is formed elsewhere. After three days of incubation, the embryos have a heartbeat and develop small blood vessels, which can be used to distinguish between male and female embryos [10,11,35,46,55]. On the fifth day of incubation, the gonads of chicken embryos begin to morphologically differentiate [56]. By the end of the eighth day of incubation, male and female embryos can be distinguished by morphological differences between testes and ovaries. On day 9 of incubation, the estrone sulfate in the allantoic fluid can be measured to determine the in ovo sex of chicken eggs [4]. After the 10th day of incubation, the allantoic fluid continues to enlarge, and feathers and feather tracts become visible. On the 11th day of incubation, the feather color difference between male and female breeds can be determined through spectral methods. On the 14th day of incubation, the claws begin to form, and the embryo fully resembles a chick. Small soft feathers cover its body, which allows for accurate sexing of chicken eggs, as male and female chicks have different feather colors [17].

In terms of the mechanism of sex differentiation, the sex of fertilized eggs can be distinguished according to hormone concentrations. Sex-targeted markers can be screened by standard assays, such as PCR and ELISA, from metabolites in the amniotic fluid, allantoic fluid, egg yolk, embryonic fluid, and serum. Extensive measurements and analyses have identified markers associated with male and female physiological secretions in chicken embryos. The concentration change trends of six steroid hormones in the serum of embryonic eggs during incubation was investigated, including estrone (E1), estradiol (E2), estriol (E3), testosterone (T), androstenedione (A4), and dihydrotestosterone (DHT) [57]. The results revealed that all hormones contributed to sex differences in embryonic eggs and that the ratio of E1 to E2 showed different trends, with the ratio increasing in male eggs and decreasing in female eggs. Moreover, T levels were significantly higher in male eggs than in female eggs, and E2 levels varied significantly more in female eggs than in male eggs. All changes were more significant in the later stages of incubation.

The mechanisms of the complex physiological changes during the incubation of chicken eggs need to be analyzed and identified more precisely. These mechanisms can be identified by the various sensors introduced in Section 3.2.1. The following section introduces big data support, including sensing and modeling techniques, in more detail in terms of applications to the in ovo sexing of chicken eggs.

3.2. Big Data Support: From Sensing to Modeling

3.2.1. Multisensing Techniques

Sensors are generally defined as devices that are used to collect and process information. Multisensor data are widely used in agricultural and food production to assess quality and condition. Due to the complex physiological structure of eggs, the acquisition of various information by multiple sensors shows great potential in in ovo sexing. In recent years, various machine vision and spectroscopic methods have been developed for in ovo sexing of chicken eggs. Different sensors, such as regular [28,44], fluorescence spectroscopy [24,26], multispectral or hyperspectral [40,57], visible/multiband spectroscopy [1] sensors, and electronic noses, have been adopted to collect qualitative data in different dimensions. Figure 6 shows different sensors and data acquisition methods used for chicken egg in ovo sexing.

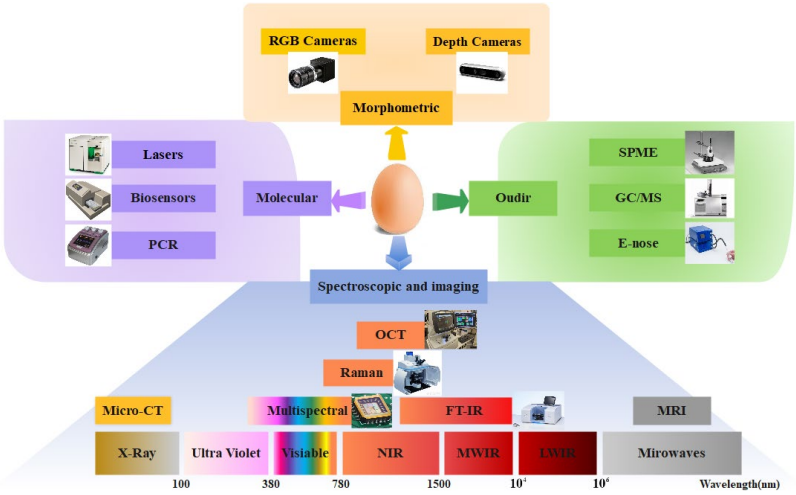

Figure 6. Multisensing techniques for in ovo sexing of chicken eggs.

Biosensors offer rapid and on-site/point-of-care detection and have been widely investigated for detecting chemicals in food and agriculture. A variety of in ovo sexing techniques have been studied, including enzyme-linked immunosorbent assay (ELISA) and PCR-based assays. The major bioprobes used in in ovo sexing are nucleic acids (DNA/RNA), proteins, and enzymes. Biosensors are also needed in PCR to amplify DNA sequences in the in ovo sexing of chicken eggs. Enzyme-based biosensors have emerged as promising tools for highly sensitive and discriminative substrate detection. Enzymatic reactions convert substrates into reaction products that can be detected by sensors. Although numerous studies have researched biosensors, only a few commercial devices are available [58]. The large-scale commercialization of enzymatic biosensors must address some significant challenges, such as the short service time of enzymes, calibration standardization, long-term stability, portability, and the elimination of interference using advanced immobilization protocols.

Spectral sensing has been extensively investigated and has proven to be an important tool in the in ovo sexing of chicken eggs. The wavelengths measured in most in ovo sexing studies cover the ultraviolet (10–400 nm), visible (400–750 nm), and near-infrared (750–2500 nm) regions of the electromagnetic spectrum. The high spectral resolution of the hyperspectral system generates a large amount of detailed spectral data, which requires a thorough understanding of the hyperspectral sensor and the measured properties.

Morphology-based in ovo sexing methods that utilize regular cameras are a more economical approach than those that apply expensive chemical analysis instruments. These approaches could be easily integrated with agricultural devices for fast, reliable, and real-time in ovo sexing of chicken eggs. The blood vessel distribution can be used to identify the sex on day 4 of incubation, while outer shape features can be analyzed before hatching, greatly reducing incubation costs. More importantly, because neither the inner membrane nor eggshell is damaged, these approaches should be accepted by most hatcheries. However, the accuracy of these methods is approximately 80–85% [16,46], which is lower than that of invasive methods. Multiple sensors could be combined to address this problem.

Eggs normally release VOCs, which are byproducts of daily physiological processes. An E-nose usually consists of a gas sensor array and an electronic pattern recognition system with multivariate statistical data processing tools. The E-nose can be trained by comparing the profile of VOCs released by female eggs with that released by male eggs. Several studies have been conducted to detect VOCs, and their results indicate that compounds of different sexes can be identified on specific hatching days; however, these approaches are still in the preliminary stage [53]. The fast response time, stability, lifetime, calibration, sensitivity, and selectivity of sensors and the standardization of gas array instruments currently limit commercial applications.

While many interesting studies have investigated one or certain types of sensors in the in ovo sexing of chicken eggs, state-of-the-art multisensor detection and data fusion technology are lacking in the literature. Multisensor systems for in ovo sexing are typically composed of various spectrometers, image sensors, and gas sensors to perform high-throughput data phenotyping of chicken eggs. The combination of multisensor and data fusion methods can use different dimensions of information to complement uncertain information and improve classification accuracy. Furthermore, a reasonable multisensor fusion algorithm can effectively improve the accuracy while reducing the impact of adverse factors. However, multisensor information fusion systems are considerably more complex than single-sensor systems, and the cost, real-time performance, applicability, and other factors must also be considered.

3.2.2. Data Processing

In terms of spectral data, the peaks and valleys in transmission and reflection spectra provide effective information. The most commonly used data smoothing methods are the moving average method, Savitzky-Golay (S-G) method, multiple scattering correction (MSC) method, and differential processing approach. Table 4 shows an analysis of the characteristics of these smoothing algorithms. The MSC method was adopted to eliminate the stray light problem and reduce noise in spectral data [41]. The second-order differential method was used to analyze the freshness of eggs [57]. The results showed that the analysis model based on the hyperspectral second-order differential method was better than that based on the first-order differential method.

Table 4. Analysis of the characteristics of the spectral preprocessing techniques used in in ovo sexing of chicken eggs.

Methods	Principles	Merits	Reference
Moving average	$x_i^* = \frac{\sum_{i=-m}^{m} X_{i+1}}{2m+1}$ *where* $2m+1$ is the width of the window	It can directly process all spectra without splitting the test and correction sets.	[57]
SG	$x_i^* = \frac{\sum_{j=-r}^{l} X_{i+j} W_j}{\sum_{j=-l}^{l} W_j}$ *where* W_j is the weighting factor in moving window smoothing (window length $2l+1$)	It is suitable for stable denoising of spectral signals and obtains better effects in terms of eliminating high-frequency noise.	[57]
MSC	$x_i^* = \frac{\overline{X_i} - b_i}{m_i}$ where m_i *and* b_i denote the relative offset coefficients and translations of X_i after linear regression	This method eliminates the scattering effects caused by uneven distributions.	[41]
Differential algorithm	$x_i^* = \frac{X_{i+j} - X_j}{g}$ where G is the differential width	This method eliminates the effects of background interference and highlights changes in spectral patterns.	[57]

After smoothing, the spectral data contain considerable spatial, spectral, and radiation information. In most studies, specific variables selected based on certain wavelength ranges result in better-performing models than using the full wavelength range. To remove the large amount of redundant data, one or more spectral feature extraction algorithms can be applied to improve the accuracy of the chicken egg sexing model. Some existing spectral feature extraction algorithms include principal component analysis (PCA), independent component analysis (ICA), the successive projections algorithm (SPA), and the competitive adaptive reweighted sampling algorithm (CARS). Table 5 shows a comparative analysis of the feature extraction algorithms used in chicken egg in ovo sexing.

Table 5. Comparative analysis of feature extraction algorithms used in in ovo sexing of chicken eggs.

Methods	Characteristic	Application Scope	References
PCA	Transforms large and highly correlated spectral data into feature information with fewer dimensions.	Suitable for multifactor spectral feature extraction, as this approach ensures that the main feature information is retained.	[11]
ICA	Decomposes the observed mixed signal into statistically independent components, which can reduce the dimension by eliminating redundant information in the original data.	Utilizes higher-order statistical information, which is more conducive to decomposing the observed signal.	[57]
SPA	Extracts several characteristic wavelengths in the full wavelength band to eliminate redundant information in the original spectral matrix.	Eliminates redundant information in the original spectral matrix and can be used to screen spectral feature wavelengths.	[35,41]
CARS	The wavelength points with large absolute values of regression coefficients in the PLS model are selected by the ARS technique, which can effectively identify the optimal variable combinations.	Addresses the problem of variable combination explosion in the variable selection process.	[35,41]

PCA was applied to study the spectral differences between male and female eggs in the blood and shell membrane due to the high degree of collinearity between the spectral data variables [11]. In another study, PCA was used to train data and obtain independent variables [17]. ICA was adopted to reduce the dimensionality of chicken egg hyperspectral data. Five characteristic wavelengths, 571, 614, 661, 691, and 716 nm, were selected [57]. The SPA algorithm was used to filter spectral data in the ultraviolet-visible light band (360–780 nm) and obtain nine wavelength variables [41]. The CARS variable selection method was used to reduce the dimensionality of the hyperspectral data. A total of 32 parameters were extracted, and a multiple regression model for egg freshness was established in the 500–1000 nm spectral band [57]. CARS, SPA, and the genetic algorithm (GA) were adopted to identify characteristic wavelengths in spectral data ranging from 500 to 900 nm [35].

The most widely used methods for processing spectroscopy data are PCA, CARS, and SPA. Several preprocessing and feature extraction algorithms have been developed to improve the adaptability of the algorithm to actual data collection environments and scales, thus eliminating the influence of other factors on the spectrum and ensuring the accuracy of the data.

3.2.3. Modeling and Decision-Making

In the last decade, many researchers have presented detailed survey results on the applications, advantages, disadvantages, and potential of deep learning methods in agriculture [59]. In ovo sexing of chicken eggs is a typical binary classification problem. The real-time, noninvasive, and early identification advantages of spectral methods have greatly improved model efficiency. Table 6 shows several popular characterization methods for chicken egg in ovo sexing.

Seven morphology features were selected and the performance of three machine learning algorithms in predicting the sex of quail eggs was compared [44]. A Canny edge was used to obtain morphological measurements. The results show that all three models based on the two-feature combination of shape index and eccentricity have higher classification rates than models with more features. The Gaussian naïve Bayes accuracy was higher than that of the logistic regression (LR) and quadratic support vector machine (SVM) approaches, obtaining an accuracy of 85.14% for males and 80.16% for females, with an average accuracy of 82.88%.

The average spectral curves of the middle part of eggs on day 10 to sex chicken eggs was collected to predict the in ovo sex using SVM, partial least-squares-discriminant analysis (PLS-DA), and artificial neural network (ANN) approaches [40]. The ANN model achieved the best gender identification accuracy, with accuracies of 88.14% (female) and 82.86% (male). Photodiodes was used to measure the average light transmittance through eggs on days 6 to 19 to determine the light transmittance of the eggs [36].

Table 6. Characterization of chicken egg in ovo sexing methods.

Type	Model	Methods	Accuracy (%)	Reference
Linear	SVM	Hyperspectral imaging	Modeling: 80.65 Validation: 75	[40]
		Blood vessel distribution	Modeling: 100 Validation: 63.33	[46]
	Kernel Naïve Bayes	Outer shape of the eggshell	81.51	[44]
		Outer shape of the eggshell	82.88	[44]
	PLS-DA	Hyperspectral imaging	Modeling: 72.58 Validation: 75	[40]
		Vis-NIR	99.05	[38]
Nonlinear	GA-BP	Blood vessel distribution	Modeling: 99.73 Validation: 82.80	[46]
	GA-ELM	Blood vessel distribution	Modeling: 100 Validation: 87.14	[41]
	ANN	Hyperspectral imaging	Modeling: 88.14 Validation: 82.86	[40]
	DBN	Blood vessel distribution	Modeling: 100 Validation: 83.33	[46]
	CNN	VIS/NIR spectra	Modeling: 93.36 Validation: 93.83	[35]

Linear discriminant analysis (LDA), LR, SVM, and K-nearest neighbor (KNN) approaches have been used to discriminate between male and female chicken eggs. The results showed that male chick embryos had higher opacity than female embryos (p value < 0.05). Moreover, various classification methods (DA, LR, SVM, and KNN) have been used to discriminate between male and female embryos. The results indicate that male and female embryo groups could be distinguished with an 84.0% accuracy using the LDA and LR models.

The composition of VOCs from male and female embryos of three different breeds was analyzed in the early hatching period (days 0–9) [60]. Random forest classification (RFC), canonical discriminant analysis (CDA), and multilayer perception (MLP) were applied for noninvasive detection of egg sex based on VOCs acquired by SPME-GC-MS. The prediction of egg sex using artificial neural network techniques on day 5 of incubation achieved accuracies of 59.5% (females) and 61.1% (males).

CARS and SPA algorithms were applied to select the characteristic wavelengths in a two-dimensional information matrix [35]. A four-layer convolutional neural network was constructed to discriminate fertility information in prehatched bred duck eggs. After the feature wavelengths were selected using the CARS or SPA algorithm, the best results were obtained using a convolutional neural network, and the performance was significantly better than that of traditional machine learning methods. The model based on the SPA algorithm and the CNN network achieved accuracies of 93.36%, 93.12%, and 93.83% on the training set, development set, and test set, respectively.

In recent years, deep learning techniques have been successfully applied to various computer vision and image processing tasks in poultry monitoring [61]. Deep learning-based approaches, including Faster R-CNN [62], You Only Look Once (YOLO) [63], and the single-shot multibox detector (SSD) [64], have been applied to object detection in recent years. These deep learning models can also be applied in morphology-based in ovo sexing methods based on the abundance of data. Furthermore, recent studies have successfully applied deep learning approaches in the high-throughput quantitative field of computer vision. High-performance computing (HPC), big data analytics, and data mining for computationally intensive tasks require high operational efficiency and acquire more accurate prediction results through decision support systems.

3.3. Commercialization and Practical Applications

The application of robot technology in the poultry field has increased rapidly in recent years. To date, only a few automatic in ovo sexing systems are commercialized and in use by the poultry sector. Some require the sampling of embryonic cells or embryo-derived cells, while others rely on the sampling of extra-embryonic fluids such as the allantoic fluid.

TeraEgg was created by Ovabrite and Novatrans to detect sex and fertility by analyzing VOCs based on terahertz (THz) signals during the chicken embryo development process. THz is a novel sensing technology with proven applications in food and agriculture [65,66]. THz spectroscopy also has potential for the detection of VOCs via identification and quantification by combinations of chemometric and spectroscopy methods. A noninvasive and preincubation approach to qualitative and quantitative analyze VOCs to achieve high-efficiency chicken egg in ovo sexing must be developed. When the measured substance is at trace levels, the weak changes in amplitude and frequency in the resonance spectrum are difficult to capture with conventional techniques. Metamaterials are a novel class of functional materials that are designed according to unique micro- and nanoscale patterns or structures to amplify detected terahertz wave signals and effectively improve the detection sensitivity [67]. However, these methods only quantitatively detect the amount of the measured substance. Moreover, they are limited in terms of specificity identification and can distinguish only among substances with known spectral properties and good resonance specificity. Therefore, ensuring that the substance to be measured has characteristic absorption peaks in the terahertz band is a prerequisite for the effective detection of sex markers in chicken embryos using terahertz metamaterial technology. A terahertz molecular sensor with a nanodot array sensing chip was designed to identify progesterone and 17α-OH-progesterone with minimum detection limits of 0.6 ng and 0.9 ng, respectively [68]. Trace-enhanced detection can be achieved with the help of microfluidic-metamaterial structures while detecting trace amounts of allantoic fluid samples. The design of nanogap antenna array metamaterials and microfluidic control techniques for terahertz-specific enhanced structures of gender markers is another important research direction.

In in ovo gender determination, PLANTegg has been developed and combined for a PCR-based method for gender determination of hatching eggs. The PCR technology identifies the genetic differences of the allantoic fluid. Researchers at Leipzig University studied endocrinological (hormone-based) sex identification and developed a marker for endocrinological sex identification in hatching eggs [4,23]. SELEGGT Circuit (2021), manufactured by SELEGGT GmbH (Germany), can continuously distinguish between female and male hatching eggs on the ninth day of incubation [69]. Lasers are used to create a 0.33 mm diameter hole in the eggshell, and a minimal amount of allantoic fluid is extracted based on differential air pressure sampling. The allantoic fluid is placed into a prepared marker that reacts to estrone sulfate by changing color. By measuring the level of estrone sulfate only present in females, the fully automatic SELEGGT Circuit can classify sex at an analysis rate of one hatching egg per second and obtains an accuracy of 98.5%, with the throughput of approximately 1 to 3000 eggs/hour. Additionally, In Ovo, a company in Leiden (the NL), developed an autonomous in ovo sexing machine known as Ella that analyzes the estradiol content in the allantoic fluid on day 9 of incubation via a Sciex Echo®Mass Spectrometer [70]. The Echo®MS technology provided by Sciex determines the concentration of the biomarker within one second and the whole system throughput is approximately 1 to 1500 eggs/hour.

In the development of non-invasive in ovo sexing systems, Agri Advanced Technologies GmbH has developed CHEGGY, which applies hyperspectral imaging to detect the gender of chicken eggs on the 13th day of incubation based on the sex-linked feather color [71]. With optimum conditions, a sexing accuracy of 98.8% can be achieved (average sexing error rate 4.1%). On the other hand, EggXYt is developing a genome editing technology for pre-incubation sex identification of embryonic chickens. Genetically edited using the tool CRISPR-Cas9, the male embryos fluoresce when illuminated with a fluorescent light through the shell [72]. It has shown a high potential and efficiency, but the ethical and

social acceptability of such approaches remains very poor. Table 7 shows several in ovo sexing systems in marketing.

Table 7. Several in ovo sexing methods in marketing.

Principle	Incubation Day	Sample	Technique	Invasiveness	Precision	Capacity	Marketing
Chromoso-mic	9 d	Allantoic liquid	PCR on cells suspended in allantoic fluid	Yes	97-99%	3000/h	PLANTegg
Molecular	9 d	Allantoic liquid	Determination of estrone sulphate	Yes	98%	SELEGGT: 3600/h; In Ovo: 1500/h	SELEGGT, In ovo
Physiological	13 d	Whole egg	Hyper-spectral imaging via feather color	No	95%	20,000/h	Agri Advanced Technologies
Genome editing	0 d	Whole egg	Imaging by fluorescence of a molecule produced by males after editing	No	100%	/	EggXYT

A fully functioning robotic in ovo sexing system should involve at least the following functions: recognition and localization of individual eggs, gender recognition, detection and removal of male eggs, and decision support for managers. A robotic system for in ovo sexing of chicken eggs faces similar and additional challenges to those faced by existing agricultural robots. Scientists and engineers across a variety of disciplines, such as biology, agricultural engineering, robotics, software architecture, intelligent systems, and hatchery egg management, have actively contributed to this field. The eggshell prevents microbial and other infections by forming a barrier between embryos and sensors. Most molecular- and spectral-based methods require perforations, which allow microbes such as bacteria to invade the embryo. This may be the main reason preventing the commercialization of these chicken egg in ovo sexing approaches. Noninvasive approaches that can easily be integrated in early embryonic stages may be more acceptable to farms.

The introduction of in ovo sexing technology in hatcheries implies that producers will have to renovate facilities, reorganize logistics, and employ more workers. For a large-scale egg hatchery, several hundred thousand eggs need to be hatched every day. It is worth noting that half of them are unwanted male breeding eggs. The extra incubation cost will be more than 100 million per year if the incubation cost is RMB 2.5 per egg. In this case, large hatcheries prefer to replace automated systems and do not consider the cost. Although small hatcheries need such equipment as well, they also need to pay more attention to cost benefit to reduce making consumers pay more money for eggs.

3.4. Prospective

To date, most techniques are not effective in determining the sex of embryos on the day of egg laying. They require (1) removal of eggs from the incubator for sexing, which may affect hatching rates; (2) in some methods, an open hole in the breeding egg to extract a sample for testing, increasing the risk of infection; (3) re-hatching female eggs, which may increase the risk of embryonic mortality; (4) culling male eggs and using them for quality feed or other purposes, and managing male chicks if the method is not 100% reliable. It should be noted that these solutions are still very marginal in terms of production or remain untested and are not the dominant production model. No one knows if these production models will expand or remain a niche market in the future. What also seems imminent is that there will be increased costs associated with the changes, which may only be partially offset by efficiency gains. However, it seems that some changes are inevitable.

Towards a practical and marketable method, the in ovo sexing methods must be precise (above 98%), fast (over 20,000 eggs per hour), and cheap. Additionally, they should be non-invasive and have no detrimental consequences on the hatchability and the viability of the chicken. For instance, the spectroscopic measurement technology does not require

expensive consumables, which makes this technique significantly less expensive than other methods and, most importantly, is more environmentally friendly. Finally, the in ovo sexing must be done before male embryo feel pain and return to the food chain. Future research and development should aim to test and commercialize in ovo sexing methods according to real-world farm conditions. High-performance extraction, robust industrial engineering, and compatibility with all setter trays would enable high-throughput processes that can be modularly scaled to any hatchery.

4. Conclusions

The present paper reviews and summarizes key techniques for in ovo sexing of chicken eggs, including molecular-, spectral-, acoustic-, morphology-, and VOC-based technologies. Most of these methods show good potential for sexing eggs before hatching. The evaluation of hormone concentrations or other biomarkers in the allantoic fluid of chicken eggs has been proven to be a robust tool for in ovo sexing of chicken eggs when processing a large number of egg samples. In terms of spectral-based techniques, most scholars have explored the potential of NIR and hyperspectral methods for in ovo sexing of chicken eggs, and the spectral multiband fusion method needs to be validated for rapid and noninvasive detection. Acoustic-based in ovo sexing of chicken eggs is still far from practical application. Morphometric methods collect and analyze eggshell outer shape data or image inner blood vessels and have not yet considered other conditions, such as variety and the physiological index of the hen. VOC profiling is a new research method that has been developed in recent years that should be studied in-depth according to qualitative and quantitative analyses of the differences in the composition of VOCs from chicken eggs on different days of incubation. The concerns about these technologies in the poultry industry include the accuracy, cost-efficiency, and recognition speed. Noninvasive approaches are practical for large-scale applications due to economic aspects and low acceptance by farmers. It is of great value to develop innovative chicken egg in ovo sexing methods, improve existing methods and engineering approaches, and provide early sex identification methods during the chicken egg incubation period.

Further research is needed to decrease the cost of sensors, improve the detection limit and classification accuracy, build a multiband database, and improve system performance and software applications in the poultry industry. More chicken egg in ovo sexing applications are expected with the development of new, more powerful spectral sources and sensors with increased sensitivity. Moreover, more machine and deep learning image processing and modeling methods, such as XGBoost and CNNs, should be applied in future research. Furthermore, these techniques should be used before a chick embryo develops its sensory nervous system, which occurs on approximately the seventh day of incubation, to ensure that the culling of the egg does not produce negative reactions. Some commercial examples autonomously sexing chicken eggs include Sellegt and Ella. In ovo sexing methods, including noninvasive sampling, assessing the in ovo environment, measuring egg quality, tracking physiological changes, and identifying and removing male eggs, must be researched further. Fully integrated systems for reliable and real-time in ovo sexing of chicken eggs can be commercialized to hopefully reduce the cruel mass culling of male baby chicks.

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