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Contactless in ovo sex determination of chicken eggs

Abstract: We resume the research for contactless in ovo sex determination of the domestic chicken, which has the final aim to provide an alternative to day-old cockerels culling in the layer industry. In ovo Raman and fluorescence spectroscopy of blood of eggs incubated until day 3.5 enables correct sexing rates over 90% barely affecting the hatching rate. Full automatization of the processes to guarantee high sexing speed and fulfill industrial demands is required to allow transferring the technology inside the hatcheries in the next future.

Keywords: Raman spectroscopy, fluorescence, in ovo sexing, day-old chick culling

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1 Introduction

Male birds of egg-laying hen strains are not suited for meat production. As they have no commercial value, they are culled immediately after hatching, rising concerns of animal welfare. Therefore, there is urgent need for methods of sex determination applicable already during egg incubation. [1]

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Classification of cell properties by optical spectroscopy is possible based on biochemical composition, and the sex may be spectroscopically retrieved from genetic and metabolic differences. In most animals including birds, the sex information is genetically encoded. For instance, cells of male domestic chickens possess larger chromosomes, enabling sexing by optical spectroscopy based on different DNA amount.

Adult birds were sexed by UV resonance Raman spectroscopy and IR absorption spectroscopy of cells extracted from the feather pulp based on DNA spectral features. [2, 3] IR spectroscopy was also applied for sexing of nonincubated eggs, where the germinal disk carries the genetic sex information: sexing was performed by addressing the DNA content of blastoderm cells. [4] In incubated eggs, all embryonic and extra-embryonic tissue derived from the germinal disk contain the sex information. For instance, spectroscopic analysis of blood offers high potential for in ovo sexing. Avian erythrocytes are nucleated and carry the genetic sex information. Moreover, analysis of blood can retrieve metabolic differences: also before onset of endocrine sex differentiation, developmental differences between male and female embryos affect blood composition.

Optical methods for in ovo sexing are contactless and directly applicable in situ. They may provide real-time sexing, without the risk of contamination and the need of waiting for the results of chemical or genetic analyses on egg tissue or fluids. Therefore, they have clear advantages when compared to standard approaches for in ovo sexing based on hormone or DNA analysis on extracted samples of egg material. Moreover, optical spectroscopy could improve animal welfare when performed before day 7 of incubation, which is the onset of embryo sensitivity to external stimuli [5]. Additionally, sexing at early stage of incubation would spare the energy required to incubate the unwanted "male eggs". Therefore, optical spectroscopy has the potential to become a technique used for in ovo sexing in layer hatcheries.

Here we resume the biological features underlying egg sexing by in ovo spectroscopic analysis of embryonic blood, and indicate a route towards deployment in poultry industry.

2 In ovo sexing by spectroscopy of embryonic blood

The development of the chicken embryo lasts 21 days and starting on day 3 of incubation the vitelline circulation is developed to some extent, enabling spectroscopic analyse of the circulating blood. At this incubation stage, the embryo is 5 to 7 mm in size, while the vascularized area of the yolk sac reaches a diameter of about 3 cm. As the egg shell is not transparent, a window some millimetre large is required in order to optically access a vessel. The spectra can be acquired under a microscope from one of the main vitelline vessels, preferably larger than 100 µm (**Figure 1**).

The use of NIR laser excitation with λ = 785 nm and P ≅ 200 mW, in conjunction with an objective with high numerical aperture, enables acquisition of the backscattered radiation spectrum within some seconds The measurement does not induce damage to the blood vessel wall (which is transparent in the visible and NIR range), or blood clotting. Thermal damage of erythrocytes is avoided, as they move with high speed driven by the heart and remain in the laser focus for a fraction of second. [6]

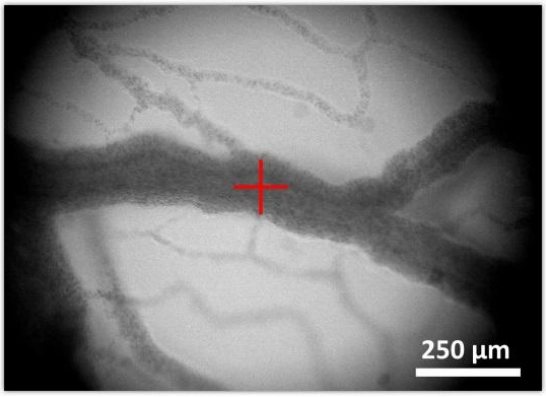

Figure 1: Vitelline vessels as visualized under the Raman microscope; the red cross indicates a suited measurement point located in a vessel larger than 100 µm.

2.1 Raman spectroscopy

In ovo sexing by Raman spectroscopy was investigated on white layer chicken eggs at day 3.5 of incubation. [6] The spectrum of embryonic blood is complex and most of the bands originate from haemoglobin. After baseline subtraction to remove the fluorescence background and normalization, the mean spectra of male and female eggs appear very similar. However, the difference spectrum highlights small differences in the range 600–1500 cm^{-1} (**Figure 2**).

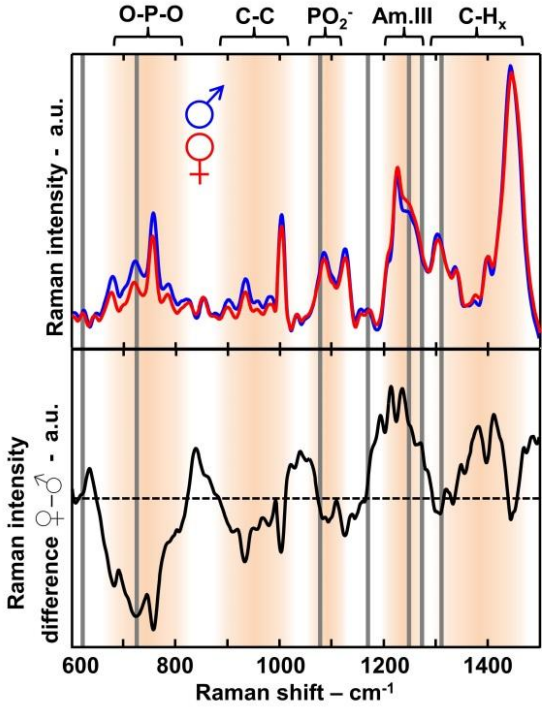

Figure 2: Raman spectra of male and female blood after baseline correction and vector normalization in the range 600-1500 cm^{-1} (mean of 165 spectra), and difference spectrum showing the bands that carry sex information; vertical lines indicate the bands retrieved by feature selection and used for classification.

Negative differences in the ranges 700–800 and 900–1000 cm^{-1} are assigned to phosphodiester linkage stretching vibrations of nuclei acids and C–C stretching modes, respectively. Phosphodioxy stretching vibrations in nucleic acids are also reported at ~1100 cm^{-1}. Amide III bands of proteins are in the range 1200–1300 cm^{-1}. Differences at 1300 and 1450 cm^{-1} are assigned to CH_x deformation modes of lipids and proteins. Therefore, Raman spectroscopy indicates that the difference between sexes is not only genetic, based on higher DNA content in male cells, but the overall blood biochemistry is sex-dependent already at early development stages before endocrine differentiation.

A refined classification strategy, based on iterative selection routine and optimization of the training set, exploited at best these differences. The selection routine retrieved the spectral regions providing sex-related information. For instance, bands associated to nucleic acids were selected at 724 and 1071-1193 cm^{-1}. Bands assigned to proteins were further selected at 616, 724, 1071-1093, 1159-1180, 1237-1257, 1262-1284 and 1303-1324 cm^{-1}. After feature selection, each spectrum was re-expressed as sub-set of intensity values, which were used for classification by linear discriminant analysis. With this approach, 90% of the spectra were classified correctly. The transferability of the classification function was verified on data acquired in an

independent experiment, obtaining a correct sexing rate of 90% as well. Classification errors are likely related to intrinsic variability of blood biochemistry, while presence of yolk and albumen contributions in the spectra due to variations of vessel diameter and focal position does not influence the classification.

2.2 Fluorescence spectroscopy

During analysis of raw Raman spectra, we observed that the mean fluorescence intensity was significantly different between sexes, although a large overlap existed (**Figure 3**). Therefore, the source of this fluorescence and the possibility to exploit it for sex determination were further investigated [7].

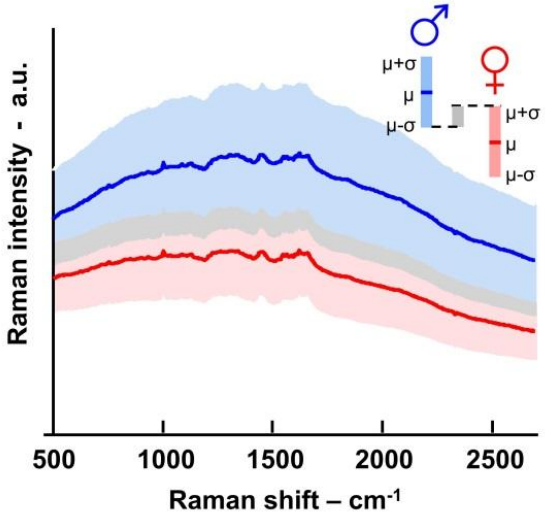

Figure 3: Backscattered spectra of male and female blood measured in ovo (mean and SD of 380 eggs), displaying the fluorescence with superimposed Raman signals.

First of all, it was observed that only the red blood cells display intense fluorescence, while all other embryonic and extraembryonic fluids and tissues give a Raman spectrum almost free from background. Furthermore, the temporal evolution of fluorescence was studied until day 12 of incubation. An increase of fluorescence was observed until day 8. The time course of fluorescence was found in agreement with haematocrit increase of observed during erythropoiesis, and haemoglobin increase during erythrocytes maturation. Therefore, the results indicated that haemoglobin is the source of the observed NIR fluorescence.

Fluorescence time course was further analysed for both sexes. Male and female egg fluorescence exhibited the largest difference at day 3.5, while the intensity difference progressively declined afterwards. At this point of the incubation, the fluorescence spectra of male and female blood displayed different spectral shape. After day 4, the fluorescence intensity of males remained higher, but the different spectral shape was rapidly reduced and totally lost by day 5. After day 9, also fluorescence intensity of both sexes became similar. Higher fluorescence of male blood at early incubation stages was related to differences in erythropoiesis between sexes.

The spectral differences existing in the backscattered spectra of blood at day 3.5 of incubation were further investigated with principal component analysis (PCA) (**Figure 4**). The first and second principal components (PC) describe the variance of fluorescence intensity and spectral shape, while higher components only describe the variance of Raman signal. For instance, PC1 loading vector represents the mean spectrum and includes both fluorescence and Raman scattering signals. PC1 score is higher for males and accounts for higher fluorescence intensity. PC2 loading vector represents a broad fluorescence signal centred at ~1800 cm^{-1}. PC2 score is higher for males and the fluorescence described by this component constitutes a spectral feature characteristic of male blood.

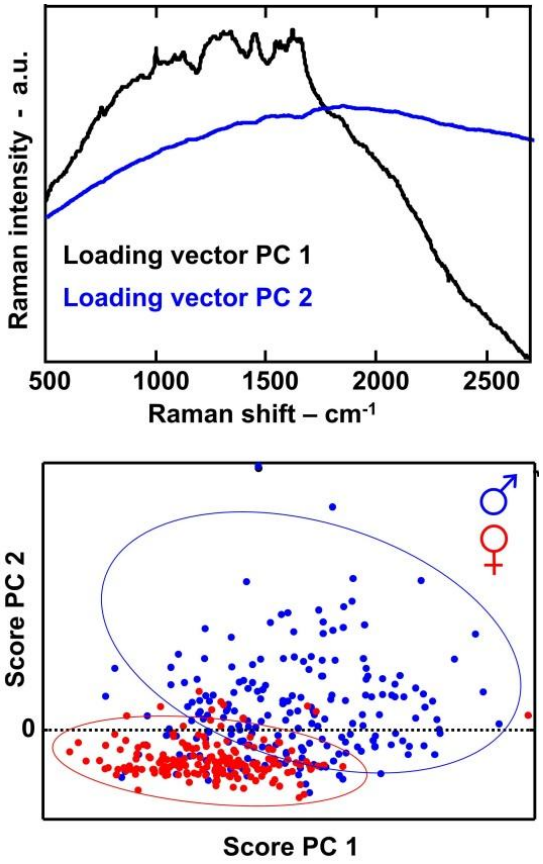

Figure 4: PC1 and PC2 loading vectors and scatter plot of scores retrieved with PCA analysis.

The difference of intensity and spectral shape at day 3.5 of incubation can be exploited for in ovo sexing. A classification based on fluorescence only (i.e., PC1 and PC2 scores) enabled sexing with correct rate up to 85%, limited by the overlap of the data. On the other side, the sexing correct rate increased over 90% by including in the classification the higher PCs describing the variations of the Raman spectra.

3 Developments towards industrial implementation

The development of an automated system performing all steps needed for sexing by optical spectroscopy is a key step towards industrial exploitation. The automatization has to include egg shell windowing, as well as sealing of female egg shells to allow continuing the incubation until hatching.

Shell windowing by CO_2 laser enables fast and clean removal of a small portion of shell without damage of underlying egg structures. As high power CO_2 lasers coupled with fast beam scanning systems exist, laser windowing of an egg can be performed in less than a second. The lift-off of the shell window should be automatically performed without damage of the embryo or of vitelline extraembryonic circulation. High quality sealing of the shell window of "female eggs" with a biocompatible material is also mandatory: as windowing must be performed at the egg pointed end in order to access the vitelline vessels, and as the eggs are incubated with the pointed end downward, imperfect sealing leads to loss of egg fluids and embryo death.

Laboratory experiments showed that it is possible to avoid reduction of hatching rate whenever the overall egg handling is correctly performed. For instance, CO_2 laser windowing was done on the nonincubated eggs, by creating just a breaking line in the shell, and gently removing the shell window with a scalpel at day 3.5 of incubation, immediately before the optical measurement. Afterwards, windows were sealed with medical grade adhesive tape. Also in case of large windows with diameter up to 12 mm, hatching rate losses were limited to few percent. [6]

For the spectroscopic measurement, a camera-based system shall enable both automatic vessel selection and setting of the laser focus inside the blood vessel. Careful selection of excitation laser source, focalisation and collection optics as well as of efficient spectrometers should enable to reduce the measure duration down to a few seconds without affecting correct sexing rate. Parallelization of several spectroscopic systems might be further realized to comply with the production need of large hatcheries.

Exploitation of multiple sex information contained in the fluorescence and in the Raman spectra appears crucial at this point of the research to further improve the classification and obtain accuracy close to the one of day-old chick sexing. Fluorescence intensity, fluorescence spectral shape and Raman scattering shall be all included in a multi-parameter classification strategy that better complies with intrinsic data variability.

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Author's Statement

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References

[1] Bruijns MRN, Blok V, Stassen EN, Gremmen HGJ. Moral "lock-in" in responsible innovation: the ethical and social aspects of killing day-old chicks and its alternatives. J Agric Environ Ethics 2015; 28:939–960.

[2] Harz M, Krause M, Bartels T, Cramer K, Rösch P, Popp J. Minimal invasive gender determination of birds by means of UV-resonance Raman spectroscopy. Anal Chem 2008; 80:1080–1086.

[3] Steiner G, Preusse G, Zimmerer C, Krautwald-Junghanns ME, Sablinskas V, Fuhrmann H, Koch E, Bartels T. Label free molecular sexing of monomorphic birds using infrared spectroscopic imaging. Talanta 2016; 150:155–161.

[4] Steiner G, Bartels T, Stelling A, Krautwald-Junghanns ME, Fuhrmann H, Sablinskas V, Koch E. Gender determination of fertilized unincubated chicken eggs by infrared spectroscopic imaging. Anal Bioanal Chem 2011; 400:2775–2782.

[5] Aleksandrowicz E, Herr I. Ethical euthanasia and short-term anesthesia of the chick embryo. Altex 2015; 32:143–147.

[6] Galli R, Preusse G, Uckermann O, Bartels T, Krautwald-Junghanns ME, Koch E, Steiner G. In ovo sexing of domestic chicken by Raman spectroscopy. Anal Chem 2016; 88:8657–8663.

[7] Galli R., Preusse G, Uckermann O, Bartels T, Krautwald-Junghanns ME, Koch E, Steiner G. In-ovo sexing of chicken eggs by fluorescence spectroscopy. Anal Bioanal Chem 2017; 409:1185-1194.