

uses of such cells, embryos, and animals. This publication particularly relate to GFP in mammalian cells.

Doran T. et al., generally describes a strategy to differentiate between males and females pre-hatch, by adding a biological marker, the GFP reporter gene, to the sex chromosome, in ASAP Animal Production 2016, Adelaide. The integration of the GFP reporter gene into the sex chromosome of chicks using the CRISPR technology was also described in a presentation at IWRAB-II, in Brasilia, in 2014. However, as this general publication discloses that the labeled chromosome is visualized *in ovo* by exposing the egg to UV light, due to the auto-fluorescence of the egg, no detectable signal is expected.

Thus, effective and non-invasive methods for sex identification during the egg stage, prior to the hatching of the chick are currently not available. There is therefore a long-felt need for a method enabling accurate and safe sex identification of the embryos in unhatched eggs.

SUMMARY

In one aspect, the present disclosure refers to a method of gender determination of avian embryo in an unhatched egg comprising the embryo within a structurally integral shell, the method comprising the step of: (a) providing at least one transgenic female avian subject comprising at least one exogenous reporter gene integrated into at least one position or location in at least one of gender chromosomes Z or W, wherein said reporter gene is a red fluorescent protein (RFP); (b) obtaining at least one unhatched egg comprising said embryo with a structurally integral shell, from said transgenic female avian subject; and (c) determining in said embryo within said structurally integral shell of said unhatched egg if at least one detectable signal is detected, wherein detection of said at least one detectable signal indicates the expression of said at least one reporter gene in said embryo within said structurally integral shell of said unhatched egg, thereby the presence of said Z chromosome or W chromosome in said embryo, thereby determining the gender of an avian embryo in an unhatched egg comprising the embryo within a structurally integral shell.

In another aspect, the present disclosure refers to an avian female transgenic subject comprising at least one exogenous reporter gene integrated into at least one locus in at least one of gender chromosomes Z or W, wherein said reporter gene is an RFP, and wherein the expression of said at least one reporter gene is suitable for identification of a gender of an avian embryo in an unhatched egg comprising the embryo within a structurally integral shell.

In another aspect, the present disclosure refers to an avian cell comprising at least one exogenous reporter gene integrated into at least one locus in at least one of gender chromosomes Z or W, wherein the expression of said at least one exogenous reporter gene is suitable for identification of a gender of an avian embryo in an unhatched egg comprising the embryo with structurally integral shell.

In yet another aspect, the present disclosure refers to a kit comprising: (a) at least one Cas9 protein or at least one first nucleic acid sequence comprising at least one nucleic acid sequence encoding said at least one Cas9 protein; and at least one gRNA that targets at least one protospacer within at least one of gender chromosomes Z or W, or nucleic acid sequence encoding said at least one gRNA; and (b) at least one second nucleic acid sequence comprising at least one said reporter gene, wherein the expression of said at least one reporter gene is suitable for identification of a gender of an avian embryo in an unhatched egg comprising the embryo within a structurally integral shell.

A first example of the disclosure relates to a method of gender determination of avian, or avian embryo in an unhatched egg, specifically, a fertilized unhatched egg. In some specific examples, the method may comprise the step of:

First, in step (a), providing or obtaining at least one transgenic avian subject or animal comprising at least one exogenous reporter gene integrated into at least one position or location (also referred to herein as locus) in at least one of gender chromosome Z and W therefore. In a second step (b), obtaining at least one fertilized egg from the transgenic avian subject or of any cells thereof.

The next step (c) involves determining in the egg if at least one detectable signal is detected. In more specific examples, detection of at least one detectable signal indicates the expression of said at least one reporter gene, thereby the presence of the W chromosome or Z chromosome in the avian embryo. In some specific

examples, the integrated reporter gene may encode the Red fluorescent protein (RFP).

In a second example, the disclosure relates to an avian transgenic animal comprising, in at least one cell thereof, at least one exogenous reporter gene integrated into at least one position or location in at least one of gender chromosome Z and W. In some specific examples, the integrated reporter gene may encode the RFP.

In yet another example, the disclosure relates to a cell comprising at least one exogenous reporter gene integrated into at least one position or locus in at least one of gender chromosome Z and W.

In yet a further example, the disclosure provides a kit comprising:

(a) at least one Cas9 protein or any fragments or derivatives thereof, or at least one first nucleic acid sequence comprising at least one nucleic acid sequence encoding said at least one Cas9 protein; and at least one nucleic acid sequence encoding at least one guide RNA (gRNA) that targets at least one protospacer located within the gender chromosome Z or W; and (b) at least one second nucleic acid sequence comprising at least one said reporter gene.

Still further, the disclosure provides a method for determining and detecting fertilization in an unhatched egg. More specifically, such method comprises the step of:

First, in step (a), providing or obtaining at least one transgenic avian subject or animal comprising at least one exogenous reporter gene integrated into at least one position or location in both gender chromosome Z and W, in case of a female subject and into both gender chromosomes Z in a male. In a second step (b) obtaining at least one fertilized egg from the transgenic avian subject, or of any cells thereof. The next step (c) involves determining in the egg if at least one detectable signal is detected. In more specific examples, detection of at least one detectable signal indicates the expression of said at least one reporter gene, thereby the presence of the labeled maternal W chromosome or Z chromosome (in case of a female or the labeled paternal Z chromosome in the avian embryo).

These and further examples of the disclosure will become apparent by the hand of the following figures.

CLAIMS:

1. A method of gender determination of avian embryo in an unhatched egg comprising the embryo within a structurally integral shell, the method comprising the step of:
 - 5 (a) providing at least one transgenic female avian subject comprising at least one exogenous reporter gene integrated into at least one position or location in at least one of gender chromosomes Z or W, wherein said reporter gene is a red fluorescent protein (RFP);
 - (b) obtaining at least one unhatched egg comprising said embryo with a structurally integral shell, from said transgenic female avian subject; and
 - 10 (c) determining in said embryo within said structurally integral shell of said unhatched egg if at least one detectable signal is detected, wherein detection of said at least one detectable signal indicates the expression of said at least one reporter gene in said embryo within said structurally integral shell of said unhatched egg, thereby the presence of said Z chromosome or W chromosome in said embryo,
 - 15 thereby determining the gender of an avian embryo in an unhatched egg comprising the embryo within a structurally integral shell.
- 20 2. The method according to claim 1, wherein said reporter gene encodes an RFP protein having an excitation wavelength of 500-650 nm and an emission wavelength of 550-650 nm.
3. The method according to claim 1 or 2, wherein said reporter gene is integrated

25 into at least one non-coding region of the target gender chromosome.
4. The method according to any one of claims 1 to 3, wherein determining if a detectable signal is detected comprises the step of subjecting said unhatched egg comprising the embryo within a structurally integral shell to a light source.

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5. The method according to any one of claims 1 to 4, wherein said at least one reporter gene is integrated into at least one position of female chromosome Z, thereby detection of a detectable signal indicates that said embryo in said unhatched egg comprising the embryo within a structurally integral shell is a male.

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6. The method according to any one of claims 1 to 4, wherein said at least one reporter gene is integrated into at least one position of female chromosome W, thereby detection of a detectable signal, indicates that said embryo in said unhatched egg comprising the embryo within a structurally integral shell is a female.

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7. The method according to any one of claims 1 to 6, wherein said at least one reporter gene is integrated into said gender chromosome of said transgenic avian female subject using at least one programmable engineered nuclease (PEN).

8. The method according to claim 7, wherein said PEN is a clustered regularly interspaced short palindromic repeat (CRISPR) type II system.

5 9. The method according to claim 8, wherein said at least one reporter gene is integrated into said gender chromosome of said transgenic female avian subject by homology directed repair (HDR) mediated by at least one CRISPR/CRISPR-associated endonuclease 9 (Cas9) system.

10 10. The method according to claim 9, wherein said at least one reporter gene is integrated into a gender chromosome of said transgenic female avian subject by contacting or co-transfecting at least one cell of said avian subject or at least one cell introduced into said avian animal, with:

15 (a) at least one Cas9 protein or at least one first nucleic acid sequence comprising at least one nucleic acid sequence encoding said at least one Cas9 protein; and guide RNA (gRNA) that targets at least one protospacer within at least one gender chromosome Z and/or W, or at least one nucleic acid sequence encoding said at least one gRNA; and

20 (b) at least one second nucleic acid sequence comprising at least one said reporter gene.

11. The method according to claim 10, wherein said at least one reporter gene in said second nucleic acid sequence is flanked at 5' and 3' thereof by homologous arms for HDR at the integration site.

25 12. The method according to claim 10 or 11, wherein said at least one reporter gene in said second nucleic acid sequence is operably linked to any one of a gender specific promoter, an embryonal specific promoter and an inducible promoter.

30 13. The method according to claim 12, wherein said at least one reporter gene is integrated into at least one site at gender Z chromosome locus 42172748-42177748.

14. The method according to any one of claims 10 to 13, wherein said at least one gRNA comprises the nucleic acid sequence as denoted by SEQ ID NO. 26.

35 15. The method according to claim 14, wherein said at least one reporter gene comprised within said second nucleic acid sequence is flanked at 5' and 3' thereof by homologous arms comprising the nucleic acid sequence as denoted by SEQ ID NOs. 31 and 32, respectively.

40 16. The method according to claim 12, wherein said at least one reporter gene is integrated into at least one site at gender W chromosome locus 1022859-1024215, wherein at least one of:

45 (a) said gRNA comprises the nucleic acid sequence as denoted by SEQ ID NO. 1, and said at least one reporter gene comprised within said second nucleic acid sequence is flanked at 5' and 3' thereof by homologous arms comprising the amino acid sequence as denoted by SEQ ID NOs. 4 and 5, respectively; and

(b) said gRNA comprises the nucleic acid sequence as denoted by SEQ ID NO. 2, and said at least one reporter gene comprised within said second nucleic acid sequence is flanked at 5' and 3' thereof by homologous arms comprising the amino acid sequence as denoted by SEQ ID NOs. 6 and 7, respectively.

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17. An avian female transgenic subject comprising at least one exogenous reporter gene integrated into at least one locus in at least one of gender chromosomes Z or W, wherein said reporter gene is an RFP, and wherein the expression of said at least one reporter gene is suitable for identification of a gender of an avian embryo in an unhatched egg comprising the embryo within a structurally integral shell.

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18. The avian female transgenic subject according to claim 17, wherein said reporter gene encodes an RFP protein having an excitation wavelength of 500-650 nm and an emission wavelength of 550-650 nm.

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19. The avian female transgenic subject according to claim 17 or 18, wherein said at least one reporter gene is integrated into at least one position of female chromosome Z.

20. The avian female transgenic subject according to claim 17 or 18, wherein said at least one reporter gene is integrated into at least one position of female chromosome W.

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21. The avian female transgenic subject according to any one of claims 17 to 20, wherein said at least one reporter gene is integrated into said gender chromosome of said avian female transgenic subject using at least one PEN.

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22. The avian transgenic subject according to claim 21, wherein said PEN is a CRISPR type II system.

23. The avian transgenic female subject according to claim 22, wherein said at least one reporter gene is integrated into said gender chromosome of said avian female transgenic subject by HDR mediated by at least one CRISPR/Cas9 system.

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24. The avian female transgenic subject according to claim 23, wherein said at least one reporter gene is integrated into a gender chromosome of said avian female transgenic subject, by contacting or co-transfecting at least one cell of said avian female subject or at least one cell introduced into said avian female subject with:

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(a) at least one Cas9 protein or at least one first nucleic acid sequence comprising at least one nucleic acid sequence encoding said at least one Cas9 protein; and at least one gRNA that targets at least one protospacer within at least one gender chromosome Z and/or W, or at least one nucleic acid sequence encoding said at least one gRNA; and

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(b) at least one second nucleic acid sequence comprising at least one said reporter gene.

25. The avian female transgenic subject according to claim 24, wherein at least one reporter gene in said second nucleic acid sequence is flanked at 5' and 3' thereof by homologous arms for HDR at the integration site.

5 26. The avian female transgenic subject according to claim 24 or 25, wherein at least one reporter gene in said second nucleic acid sequence is operably linked to any one of a gender specific promoter, an embryonal specific promoter and an inducible promoter.

10 27. The avian female transgenic subject according to any one of claims 24 to 26, wherein said at least one reporter gene is integrated into at least one non-coding region of said gender chromosome.

15 28. The avian female transgenic subject according to claim 27, wherein said at least one reporter gene is integrated into at least one site at gender Z chromosome locus 42172748-42177748.

20 29. The avian transgenic subject according to any one of claims 24 to 28, wherein said at least one gRNA comprises the nucleic acid sequence as denoted by SEQ ID NO. 26.

30 30. The avian transgenic subject according to claim 28, wherein at least one reporter gene comprised within said second nucleic acid sequence is flanked at 5' and 3' thereof by homologous arms comprising the amino acid sequence as denoted by SEQ ID NOs. 31 and 32, respectively.

25 31. The avian female transgenic subject according to any one of claims 24 to 27, wherein said at least one reporter gene is integrated into at least one site at gender W chromosome locus 1022859-1024215, and wherein at least one of:

30 (a) said gRNA comprises the nucleic acid sequence as denoted by SEQ ID NO. 1, and said at least one reporter gene comprised within said second nucleic acid sequence is flanked at 5' and 3' thereof by homologous arms comprising the amino acid sequence as denoted by SEQ ID NOs. 4 and 5, respectively; and

35 (b) said gRNA comprises the nucleic acid sequence as denoted by SEQ ID NO. 2, and said at least one reporter gene comprised within said second nucleic acid sequence is flanked at 5' and 3' thereof by homologous arms comprising the amino acid sequence as denoted by SEQ ID NOs. 6 and 7, respectively.

40 32. An avian cell comprising at least one exogenous reporter gene integrated into at least one locus in at least one of gender chromosomes Z or W, wherein the expression of said at least one exogenous reporter gene is suitable for identification of a gender of an avian embryo in an unhatched egg comprising the embryo with structurally integral shell.

45 33. The avian cell according to claim 32, wherein said reporter gene is an RFP having an excitation wavelength of 500-650 nm and emission wavelength of 550-650 nm.

34. The avian cell according to claim 32 or 33, being a primordial germ cell (PGC).

35. The avian cell according to any one of claims 32 to 34, wherein said at least one reporter gene is integrated into a gender chromosome of said cell, by contacting or co-transfecting said cell with:

- 5 (a) at least one Cas9 protein or at least one first nucleic acid sequence comprising at least one nucleic acid sequence encoding said at least one Cas9 protein; and at least one gRNA that targets at least one protospacer within at least one gender chromosome Z and/or W, or nucleic acid sequence encoding said at least one gRNA; and
- 10 (b) at least one second nucleic acid sequence comprising at least one said reporter gene.

36. The avian cell according to claim 35, wherein said at least one reporter gene in said second nucleic acid sequence is flanked at 5' and 3' thereof by homologous arms for HDR at the integration site.

37. The avian cell according to claim 35 or 36, wherein said gRNA comprises the nucleic acid sequence as denoted by SEQ ID NO. 26, and wherein said at least one reporter gene comprised within said second nucleic acid sequence is flanked at 5' and 3' thereof by homologous arms comprising the amino acid sequence as denoted by SEQ ID NOs. 31 and 32, respectively.

38. The avian cell according to claim 36 or 37, wherein at least one of:

- 25 (a) said gRNA comprises the nucleic acid sequence as denoted by SEQ ID NO. 1, and wherein said at least one reporter gene comprised within said second nucleic acid sequence is flanked at 5' and 3' thereof by homologous arms comprising the amino acid sequence as denoted by SEQ ID NOs. 4 and 5, respectively; and
- 30 (b) said gRNA comprises the nucleic acid sequence as denoted by SEQ ID NO. 2, and wherein said at least one reporter gene comprised within said second nucleic acid sequence is flanked at 5' and 3' thereof by homologous arms comprising the amino acid sequence as denoted by SEQ ID NOs. 6 and 7, respectively.

39. A kit comprising:

- 35 (a) at least one Cas9 protein or at least one first nucleic acid sequence comprising at least one nucleic acid sequence encoding said at least one Cas9 protein; and at least one gRNA that targets at least one protospacer within at least one of gender chromosomes Z or W, or nucleic acid sequence encoding said at least one gRNA; and
- 40 (b) at least one second nucleic acid sequence comprising at least one said reporter gene, wherein the expression of said at least one reporter gene is suitable for identification of a gender of an avian embryo in an unhatched egg comprising the embryo within a structurally integral shell.

40. The kit according to claim 39, wherein said at least one reporter gene in said second nucleic acid sequence is flanked at 5' and 3' thereof by homologous arms for HDR at the integration site.

41. The kit according to claim 39 or 40, wherein said at least one reporter gene in said second nucleic acid sequence is operably linked to any one of a gender specific promoter, an embryonic specific promoter and an inducible promoter.
- 5 42. The kit according to any one of claims 39 to 41, wherein said at least one reporter gene is adapted for integration into at least one non-coding region of said gender chromosome.
- 10 43. The kit according to claim 42, wherein said at least one reporter gene comprised within said second nucleic acid sequence is flanked at 5' and 3' thereof by homologous arms comprising the amino acid sequence as denoted by SEQ ID NOs. 31 and 32, respectively, and wherein said at least one gRNA comprises the nucleic acid sequence as denoted by SEQ ID NO. 26.
- 15 44. The kit according to any one of claims 39 to 43, wherein at least one of:
(a) said gRNA comprises the nucleic acid sequence as denoted by SEQ ID NO. 1, and wherein said at least one reporter gene comprised within said second nucleic acid sequence is flanked at 5' and 3' thereof by homologous arms comprising the amino acid sequence as denoted by SEQ ID NOs. 4 and 5, respectively; and
20 (b) said gRNA comprises the nucleic acid sequence as denoted by SEQ ID NO. 2, and wherein said at least one reporter gene comprised within said second nucleic acid sequence is flanked at 5' and 3' thereof by homologous arms comprising the amino acid sequence as denoted by SEQ ID NOs. 6 and 7, respectively.
- 25 45. The kit according to any one of claims 39 to 44, wherein said reporter gene is an RFP having an excitation wavelength of 500-650 nm and emission wavelength of 550-650 nm.
- 30 46. The kit according to any one of claims 39 to 45, for use in the preparation of at least one avian female transgenic subject comprising at least one exogenous reporter gene integrated into at least one locus in at least one of gender chromosome Z and W.